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Purification and characterization of macerans amylase

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NOTE TO USERS

PURIFICATION AND CHARACTERIZATION OF MACERANS AMYLASE

by

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INTRODUCTION

The only known homogeneous dextrins formed from starch, by chemical or enzymatic means, are the cyclic Schardinger dextrins. These crystalline dextrins are formed from starch by the action of macerans amylase, an enzyme elaborated by Bacillus macerans. They have been shown to have cyclic structures of six or more glucose units linked by α -1,4-glucosidic bonds.

An understanding of the mode of formation of these dextrins might be of value in the clarification of the configuration of starch. Studies of the actions of other characterized enzymes on starch have already proved informative here. An understanding of the type of reactions catalyzed by macerans amylase might also uncover new possibilities to be considered in the study of the natural scheme of polysaccharide metabolism.

Like the hydrolytic alpha and beta-type amylases, macerans amylase degrades starch to lower molecular weight dextrins and sugars. However, in addition, it must be capable of synthesizing an α -1,4-glucosidic bond during the formation of each cyclic dextrin molecule. Phosphorylase is also capable of synthesizing these glucosidic bonds, but, unlike macerans amylase, requires a specific cosubstrate

containing an "energy-rich" bond, glucose-1-phosphate.

Such a unique action has allowed speculation on the existence of more than one enzymic component in macerans amylase preparations. Much of this work has been directed toward purification of the enzyme to facilitate its identification as a single molecular species.

Recent introduction of the homogeneous "amylaceous" substrate, amyloheptaose, has greatly aided this investigation. This, together with glucose, maltose, and the Schardinger dextrins, has afforded possibilities for defineable substrates, upon which enzyme action could be more easily followed. The nature of the cosubstrate phenomena, in which macerans amylase requires linear glucosidic cosubstrates for reaction with purified Schardinger dextrins, has also been clarified.

This investigation has attempted to formulate a clear qualitative picture of many of the single reactions catalyzed by macerans amylase. It is believed that an understanding of the course of reaction in these limited cases, should provide a key to an understanding of the general picture of macerans amylase action on its natural starchy substrates.

REVIEW OF THE LITERATURE

Antoine Villiers (1, 2, 3) first reported the formation of a different type of crystalline dextrin from starch by the action of "butyric ferment", a crude culture of Clostridium butyricum. He demonstrated that these dextrins were unique in being nonfermentable, nonreducing to copper reagents, nonreactive with phenylhydrazine, and more resistant to acid hydrolysis than starch or other dextrins. These crystalline dextrins were formed concurrent with a decrease in iodine coloration and a change in specific rotation of the starch digest. These changes were also shown to occur in the organism-free filtrate of the "fermenting liquid". Villiers characterized the dextrins formed by specific rotation, solubility, and acid hydrolysis to glucose. He postulated their formation from starch as a transformation of the "amylaceous" material, effected by a soluble product secreted by the organism, capable of transforming starch in the absence of the organism.

In 1903, Franz Schardinger isolated a thermophilic organism which produced crystalline dextrins from starch (4). He later found a similar action in cultures of another isolate which he had named Bacillus macerans (bacillus of retting) (5). His first interest in the

macerans organism had been as "ein Aceton bildender Rottebacillus", and he had recorded bacteriological studies on the pure culture: its occurrence, morphology, nutrition, and acetone production. Subsequently, Schardinger turned to a study of the unique crystalline dextrins formed by this organism from starchy media. He showed that the crystalline dextrins formed from wheat, rice, potato, and arrowroot starches were the same (6). Finally he developed a procedure for the isolation of two different crystalline dextrins which he called alpha and beta, and characterized each by specific rotation, elemental analysis, and their unique crystalline iodine complexes (6,7). The constants which Schardinger reported for his dextrins have been replaced with more accurate figures for purer preparations, but his alpha and beta dextrins themselves have subsequently become known as "Schardinger dextrins".

Following the initial work of Villiers and Schardinger, little interest was shown in Bacillus macerans for its ability to produce Schardinger dextrins. However, many workers did investigate the organism as a fermentation source of ethyl alcohol and acetone (8, 9, 10, 11, 12, 13). After 1920, studies were reported which led ultimately to a precise bacteriological classification of Bacillus macerans and description of its morphological and physiological characteristics and its capacity for the production

of Schardinger dextrins from starch.

Villiers had early detected the existence of a soluble substance responsible for the transformation of starch to his so-called "cellosine" dextrins. After the enzymatic nature of this starch-digesting substance became apparent, it was called "macerans amylase". Recent work on Bacillus macerans has been directed toward establishment of optimal conditions for the elaboration of this amylase.

In 1930, Zacharov (13) published some morphological studies on B. macerans in which he described a purification procedure to free the macerans organism from similar contaminating bacteria and presented illustrated descriptions of the growth characteristics of the organism. Porter, McCleskey and Levine (14, 15) have further characterized B. macerans in their differentiation of facultative, sporulating, aerogenic bacilli into the "macerans" and "polymyxa" groups. In contrast to the "polymyxa" group, B. macerans was shown to give a negative Voges-Proskauer reaction, to produce acid and gas from rhamnose and sorbitol media, to show optimal growth at 42° - 45° C. but little or no growth at 20° C., and to be serologically homogeneous.

Following these classification studies, growth studies were made to determine optimal conditions for macerans amylase production by the organism. Freudenberg and Jacobi

(15) established a repeated transfer cycle for building up cultures capable of dextrin formation from starch; Blinc (17) established the optimum temperature for inoculation and incubation; Tilden and Hudson (18) determined the optimal medium and buffer concentrations; and Daniels (19, 20) demonstrated that aeration and oxygenation of growing cultures greatly increased enzyme elaboration.

The unit of macerans amylase activity as defined by Tilden and Hudson (18) has been accepted by most recent workers. It has been defined as "the quantity which will convert 30 mg. of starch to the brown-violet stage in 30 minutes at 40° C. at the optimal pH (about pH 6.0)". The "brown-violet stage" is easily detected microscopically by the change of the α-dextrin-iodine complex crystal habit of the assay digest from blue hexagons to dichroic needles.

Blinc (17) independently developed an "amylase unit of viscosity", based on the time necessary for a one-tenth volume of enzyme solution to reduce the viscosity of a standard starch solution to that of a standard sucrose solution. However, the convenience and macerans specificity of the "Tilden end point" has assured its general use in most subsequent work.

The first concentration of the active enzyme component of the bacterial filtrate was reported by Tilden and Hudson

(21). They precipitated a fraction containing macerans amylase with 37.5 percent acetone and effected a concentration by extraction with one-tenth volume of water. Blinc (17) also showed that precipitation of the bacterial filtrate with 80 to 90 percent ethanol gave a precipitate, which on solution exhibited full activity for starch dextrinization.

Tilden, Adams, and Hudson (22) subsequently achieved the highest degree of purification of macerans amylase reported: a 140-fold increase in activity per unit volume of enzyme solution. Their procedure included acetone precipitation, adsorption on aluminum hydroxide from acetate buffer at pH 4.8, elution with phosphate buffer at pH 7.6, acetone precipitation, and dialysis. Their preparations assayed approximately 30 units per mg. of solids.

Characterization of macerans amylase as a protein enzyme was attempted by several workers on enzyme preparations of varying degrees of purity. Tilden, Adams, and Hudson (22) found that, in their purest preparation, the enzyme molecules were of sufficient size to pass through a collodion membrane of 60 millimicron porosity, but were retained by a 40 millimicron membrane. This established the enzyme to be at least of relatively high molecular weight.

They also found that, in the presence of starch substrate, the enzyme was stable to heating at 50° C. for at least one hour and at 70° C. for fifteen minutes (18).

Blinc (17) found little inactivation of the enzyme up to 65° C., and only 31 percent loss in activity at 80° C. From this it may be assumed that temperature increase causes an increase in the rate of enzyme activity up to approximately 65° C. At this point, inactivation, or denaturation, proceeds at a greater rate than enzyme activity acceleration.

The influence of pH on the enzyme protein could similarly be deduced by the effects of varying pH on the rate of enzymolysis. Thus, Tilden and Hudson (18) found inactivation or denaturation below pH 4.2 and above pH 6.5. Myrbäck and Gjorling (23) obtained similar results on the enzyme in the crude bacterial filtrate. They showed an activity increase from zero at pH 4 to a maximum near pH 6 and a gradual decrease again to zero at pH 8. So, stability of the enzyme, as measured by relative activity, is greatest between pH 5 and 6 in the presence of a starch substrate.

The stability of the enzyme to drying has been found to vary with the degree of purification of the preparation. Tilden, Adams, and Hudson (22) report that their purest preparations retained full activity for many months in sterile solution at 5° C. However, acetone precipitates, dried over P_2O_5 at 5° C. and kept cold, had lost 35 percent of their

activity after seventy-nine days. Daniels (20) found that lyophilized B. macerans filtrates retained full activity after three months in the cold. This is not unexpected, since Tilden and Hudson (18) also point out that pure B. macerans cultures, or their filtrates, retain full activity for several days at 37° C., and for at least a year at 5° C. Thus, as with most enzyme proteins, stability is greatest in solution and in the less purified preparations.

These physical characteristics of macerans amylase have served to differentiate it sharply from the other amylases. The bacterial alpha-type amylase from B. polymyxa differs in its maximal pH at 6.8, and its marked inactivation on incubation at 50° C. (18). Malt alpha-amylase and takadiastase are most active at pH 4.5 to 5.5 (24); pancreatic and salivary alpha-amylases, at pH 6.8 (25); and cereal beta-amylase, at pH 6.5 to 7.0 (25). Crude takadiatase is completely inactivated by heating to 60° C. for fifteen minutes, and malt diastase, by heating to 70° C. for fifteen minutes (24). In general, beta-amylases are even more thermolabile than alpha-amylases.

Clearly, macerans amylase has been shown to be physically a distinct type of amylase. It can further be shown that its chemical behavior differentiates it even more sharply.

Characterization of the Schardinger dextrins was a necessary preliminary to studies on the mode of action of macerans amylase. In his initial work, Villiers (2) had detected two crystalline dextrins. Schardinger isolated these, which he called alpha and beta dextrins, and described their physical and chemical properties. Later, Freudenberg and Jacobi (16) reported the isolation of five distinct dextrins. These they obtained by the action of B. macerans on starch, and fractionation of the products through the varying solubilities of the dextrins and their acetates. More recent work by Freudenberg et al. (26) has reaffirmed their report of five dextrins; and identification of the third dextrin, gamma dextrin, has been substantiated by French and co-workers (27).

Degradation of the alpha and beta dextrins and their acetates provided a clue to the nature of their chemical structure. Freudenberg and co-workers showed that acid hydrolysis, acetolysis, or takadiastase digestion converted the dextrins completely to glucose (28, 29); acid hydrolysis of the methylated dextrins gave only 2,3,6-trimethyl glucose (29). Hydrolysis was accompanied by an initial rise in rotation, followed by a drop to that of glucose. At first, on the basis of this evidence, these workers postulated a straight chain structure with a levoglucosan type of

termination for the non-reducing dextrins. However, more quantitative data led to the acceptance of a cyclic molecule in which all glucose units were linked by α-1,4-glucosidic bonds (23, 30).

Scale models of such cyclic dextrins predicted that at least five glucose residues were necessary to produce a structure without tension (31). On the basis of cryoscopic measurements on the dextrins and their acetates (26, 28, 30) and X-ray analysis of the crystals (32), Freudenberg and his co-workers postulated that the alpha dextrin ring contained five glucose residues, and the beta, six. The more recent studies by French and Rundle (33) using X-ray diffraction and crystal density measurements have shown the alpha dextrin to contain six glucose residues per molecule, and the beta dextrin, seven glucose residues. These workers have named the two dextrins cyclohexaamylose and cycloheptaamylose, respectively.

Clarification of the structures of the Schardinger dextrins allowed speculation on the manner of their formation. For some time, it was a disputed question whether they were preformed in starch, or synthesized from open chains by the action of the macerans enzyme. Freudenberg (34) initially assumed the dextrins were preformed in starch because of their iodinestaining ability. Tilden and Hudson (21) also at first thought them to be true

components of starch because of their formation without the liberation of glucose or maltose. However, studies of macerans action on different amylaceous substrates showed that the cyclic dextrins were products of enzymatic synthesis.

Schardinger dextrin yields from macerans action on starch have been reported up to 55 percent of the original starch substrate by Hudson and co-workers (35, 36). Yields of 70 percent from the amylose fraction of starch have been reported by Kerr (37); Wilson, Schoch, and Hudson corroborate this and further report only 49 to 50 percent yields from the amylopectin fraction (36). Studies by Kerr on acid treated starch showed the yields of Schardinger dextrins to vary inversely with the degree of preliminary hydrolysis (38). Samec obtained Schardinger dextrins from alpha-amylase achroo dextrins, but more from the amylose achroo dextrin than from the amylopectin achroo dextrin. He found no dextrins produced from alpha-amylase erythro dextrins or beta-amylase limit dextrin (39). Kerr further reported no dextrins formed from diastase limit dextrin (26).

From the foregoing, it can be seen that cyclic dextrin formation by macerans amylase is greater from an unbranched substrate. With branched substrates the dextrin formation decreases as the end chain length decreases. Myrbäck and

Gjorling (23) supported these conclusions by correlation of the actions of macerans and beta-amylases. They pretreated samples of starch with macerans amylase and recorded the relative recoveries of Schardinger dextrins. Subsequent action by beta-amylase on these predigested samples showed a decrease in maltose production from those samples which had produced the greatest amounts of Schardinger dextrins. Thus, they concluded that macerans amylase attacks preferentially those linkages susceptible to beta-amylase action; i.e. the straight chain fraction of starch and the non-reducing end chains of the branched chain starch fraction.

While the site of macerans action on starch was being clarified, certain facts on the course of action were made apparent. Time studies of macerans action on starch have shown it to encompass three successive stages:

1. An initial rapid decrease in the viscosity of the starch substrate or "dextrinization". This rapid liquefying action of macerans amylase had been recognized early in its history. Myrbäck and Gjorling (23) showed that this initial viscosity decrease followed a rate curve identical with that of alpha-amylase. McClenehan, Tilden, and Hudson (35) obtained a similar plot. Kneen and Beckord (40) followed the "dextrinization" by conversion of the blue starch-iodine color to a red-brown dextrin-iodine color. They found the macerans action rapid, but incapable

of conversion of starch to achroo dextrins, as with the other types of bacterial amylases studied.

2. Schardinger dextrin production. The formation of Schardinger dextrins proceeds after the initial liquefaction phase. McClenahan, Tilden, and Hudson (35) have followed the yield of crude dextrins from starch and have shown it to increase gradually up to fifty percent. They have found the alpha dextrin to be formed first up to twenty percent, with a subsequent decrease in alpha, as the beta dextrin is produced. Myrbäck and Gjorling (28) have reported a complete disappearance of the Schardinger dextrins after a period of maximum yield. However, this observation has not been confirmed with purified enzyme preparations.

3. Production of fermentable sugars, or "saccharification". Myrbäck and Gjorling (23) reported an increase in maltose formation concurrent with the disappearance of Schardinger dextrins in their prolonged time studies on macerans amylase action on starch. Kneen and Beckord (40) also found a delayed production of fermentable sugars, which proceeded more slowly, and to a lesser degree, than that of other bacterial amylases with comparable dextrinizing action. All of these workers used the crude bacterial filtrates for their studies. Thus, this phase of macerans

action remains questionable; time studies with long digestion periods have not been performed with purified enzyme preparations.

Correlation of the data on the phases of macerans action is difficult because of the varying degrees of purity of the enzyme preparations used. However, general trends in changes of specific rotation and reducing values of starch digests can be noted. Villiers (3) in his early work, and later, Blinc (17), reported an initial increase in specific rotation of a starch macerans digest. They both reported a return to the initial substrate value after the maximum specific rotation had been reached. McClenahan, Tilden, and Hudson (35) have recorded only a rotational decrease during macerans amylase action on starch. This would not be unexpected, since dextrins of lower specific rotations are formed.

Blinc (17) had also reported an initial rise in reducing power during macerans dextrinization of starch, with an eventual return to the original value. McClenehan, Tilden, and Hudson (35) reported a very slight increase in reducing power, i.e. 1.35 per cent during the viscosity drop. In digests giving up to fifty percent yields of Schardinger dextrins, these authors found a maximum increase in reducing power of 1.64 percent of the theoretically available glucose. It is true that Myrbäck and Gjorling (23) found

a much greater hydrolytic action in their bacterial filtrates. However, this, together with a high degree of saccharification after longer periods of time, might point to a possible contamination of their enzyme solution with an alpha-type amylase. In general, it might be said that in work published to date the hydrolytic activity found in macerans amylase has been least where the purity of enzyme preparation has been greatest and where the digestions have been conducted for shorter periods of time.

Thus, liquefaction, Schardinger dextrin formation, and possibly saccharification have been shown to occur with liberation of at least relatively few reducing groups. This is in direct contrast to the action of the alpha and beta-amylases, with which reducing power of the substrate increases proportionately as dextrinization and saccharification proceed.

Some work has been recently reported on substrates more sharply defineable than starch. McClenahan, Tilden, and Hudson (35) had reported that their purified alpha dextrin preparation was converted by macerans amylase at least partially to a higher rotating material that exhibited slight reducing properties. This product gave no test for beta dextrin. Pure beta dextrin, in turn, was not acted upon by macerans amylase.

French and co-workers (41) have enlarged on these preliminary observations to demonstrate the reversibility of macerans amylase action. They have shown that macerans amylase is capable of synthesizing higher saccharides from both alpha and beta dextrins in the presence of certain specific "cosubstrates". Thus, alpha dextrin may be converted to higher straight chain saccharides in the presence of glucose, maltose, α-methylglucoside, sucrose, cellobiose, or maltobionic acid. Similarly, the beta dextrin may be acted upon in the presence of glucose or maltose. The longer chain products, on isolation, could in turn be converted back to alpha dextrin by macerans amylase.

Levine (42) also prepared the straight chain oligosaccharide, amyloheptaose, by controlled acid hydrolysis of beta dextrin. He characterized it by its phenylhydrazone, the potassium salt of its acid, specific rotation, alkaline ferricyanide reducing value, molecular weight determination by iodine oxidation, and demonstration of its freedom from glucose, maltose, and trisaccharide. From this heptasaccharide substrate, he showed that macerans amylase could produce alpha dextrin, glucose, maltose, trisaccharide, and other oligosaccharides without the production of additional reducing groups. Some longer chain saccharides were also produced as evidenced by a deepening of iodine coloration.

So the action of macerans amylase has been shown to be synthetic as well as degradative, apparently proceeding through the energy exchange from one α-1,4-glucosidic bond to a new similar bond. Introduction of the use of lower molecular weight, more homogeneous, substrates has laid the foundation for a more precise characterization of the mode of action of macerans amylase.

DEFINITION OF TERMS

Enzyme Purity

The relative degrees of purity of different enzyme preparations were based on the nitrogen or tyrosine values per unit activity. Nitrogen determinations were carried out by the standard micro-Kjeldahl method. Tyrosine was determined by the Folin-Ciocalten procedure (43).

By running duplicate analyses on the same sample, a straight line relationship was found between millimoles tyrosine and milligram nitrogen per unit activity. This in turn could be related to protein content, if the conversion factor from nitrogen to protein was assumed to be 6.25. Figure 1 and Table I show these relationships. In general, it was assumed that one milligram nitrogen was approximately equivalent to two micromoles of tyrosine and 6.25 milligram protein.

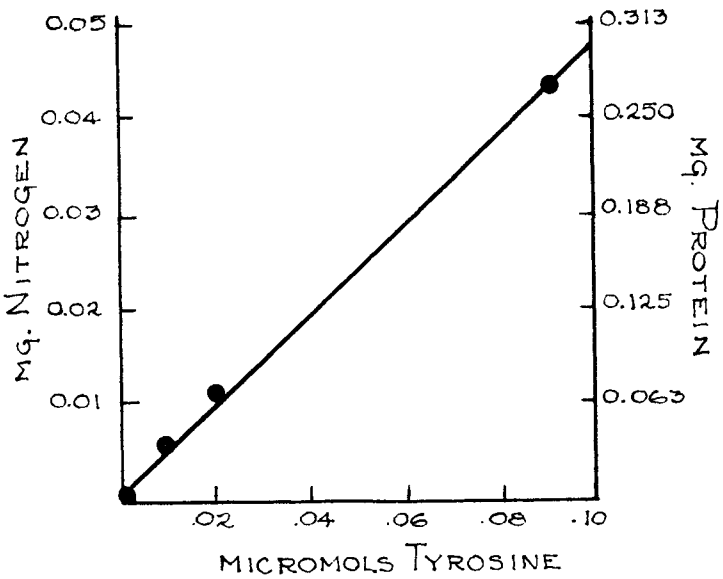

Fig. 1. Relative Nitrogen, Tyrosine, and Protein Contents of Enzyme Preparations.

Table I. Nitrogen and Tyrosine Analyses of Representative macerans Amylase Preparations.

Enzyme solution	Mg. Nitrogen per unit activity	Micromoles tyrosine per unit activity
Acetone precipitate	.0435	.09
First Alcohol precipitate	.0112	.02
Second Alcohol precipitate	.0057	.01

Macerans Amylase Activity

The method used for assay of macerans amylase activity was that of Tilden and Hudson (18). The unit of activity was based on the identification of an endpoint which is characteristic only of macerans amylase.

One ml. of enzyme solution was digested with 30 mg. of soluble starch in a total volume of 3 ml. at 40° C. At timed intervals, three drops of this digest were added to one drop of 0.1 N iodine in 0.1 M KI on a spot plate. This was mixed thoroughly and streaked on a glass slide. Examination of the streak under the microscope revealed the formation of characteristic alpha dextrin-triiodide crystals at the margin of the streak as it dried. The point at which the crystal habit first changed from blue hexagons to dichroic needles was the assay endpoint.

One unit of activity has been defined by Tilden and Hudson as that amount of enzyme which will convert 30 mg. of starch to the red-brown endpoint in 30 minutes after 40° C. at the optimal pH (about pH 6.0).

Conversion Period

The concept of conversion period for *macerans* action has been defined by French, *et al.* (27) as "that time of reaction between enzyme and substrate which, under the conditions, would be just sufficient to convert an equal weight of starch to the Tilden and Hudson end-point". The timing of enzymolyses in conversion periods, then, allows comparison of reactions of different enzymic activity, substrate concentration, temperature of incubation, and time of reaction.

This concept has been extended in this work to include the action of the salivary alpha-type amylase. Here the endpoint of the hypothetical reaction has been set at that point at which the starch-iodine color of the enzymolysate is the same red-brown as that produced at the Tilden and Hudson *macerans* endpoint. This provides a means of comparing alpha type and *macerans* amylases of equal "dextrinizing" activities.

EXPERIMENTAL PROCEDURE AND RESULTS

Preparation of Macerans Amylase

Bacillus macerans cultures

To obtain Bacillus macerans filtrates of constant amylase activity, all cultures were carried through a complete series of transfers as follows:

1. An active strain of B. macerans was plated out on China Blue-lactose agar (1 percent peptone, 2 percent agar, 0.3 percent yeast extract, China Blue indicator) and incubated at 35° C. for two to three days.

2 a. A single characteristic blue macerans colony was streaked onto a sterile potato slant (prepared by soaking the cut potato slants in 1 percent sodium carbonate for one-half hour and autoclaving for 20 minutes at 20 pounds) and incubated at 35° C. for three to five days; or

b. Macerans colonies were subcultured to 10 ml. portions of sterile glucose, lactose, or sorbitol broth (1 percent sugar or sorbitol, 0.5 percent peptone, 0.3 percent yeast extract, calcium carbonate) and incubated at 35° C. until gas evolution was observed.

3. Twenty ml. portions of sterile oatmeal medium (5 percent rolled oats, 2.5 percent calcium carbonate, and

0.25 percent ammonium sulfate, boiled to decrease foaming and autoclaved for 20 minutes at 20 pounds) were inoculated with loops of actively growing cultures from potato slants, or with the 10 ml. broth cultures, and incubated at 35° C. until active gas evolution was observed.

4. One-hundred ml. portions of potato medium (10 percent potato slices, 2.5 percent calcium carbonate, and 0.25 percent ammonium sulfate, autoclaved for 20 minutes at 20 pounds) were inoculated with the 20 ml. actively growing oatmeal cultures and incubated at 35° C. until active gas evolution was observed.

5. One liter portions of sterile oatmeal medium (prepared as in 3) were inoculated with the 100 ml. actively growing potato cultures and incubated in rotating bottles, to provide constant mixing of the cultures, at 41° to 45° C. for one week.

The filtrates from the final liter volume cultures consistently assayed three to six units per milliliter by the method of Tilden and Hudson (18).

There appeared to be no difference in macerans amylase production among different strains of B. macerans when cultured under these conditions. Strains used in these studies were B. macerans, 277 and 888.

Acetone and alcohol precipitation

The efficiency of enzyme precipitation from the bacterial filtrates by acetone and ethanol varied with different crude bacterial filtrates. It was found that the enzyme, with other proteinaceous material, was precipitated from the bacterial filtrates at acetone concentrations of 30 to 50 percent and at ethanol concentrations of 35 to 60 percent by volume.

Acetone proved to be superior for the nature of the precipitate produced. It rendered the slime-producing materials water insoluble and gave a less viscous solution on extraction with water. Treatment with acetone up to 25 percent allowed removal of most slime-forming constituents before enzyme precipitation at higher acetone concentrations.

Ethanol gave a slimy viscous precipitate which was difficult to centrifuge and impossible to filter with applied suction or pressure. However, the precipitate had the advantage of being practically salt-free. The presence of acetone-insoluble salts was found to reduce enzyme activity, by inactivation or adsorption, during concentration of the protein solutions.

Recovery of the active enzyme was found to be better after precipitation at lower temperatures. The enzyme proved to be stable in the presence of either agent at

2° C. However, the heat of hydration of both precipitants made it essential to reduce their initial temperatures so that the temperature of the protein solution did not rise above 2° C. during mixing. The most convenient and effective procedure was to precool the precipitant in a dry ice bath. This was then added with stirring to the bacterial filtrate which was immersed in a cold bath at -10° C. Total volumes were kept below four liters.

Precipitates were then centrifuged immediately in a Sharples Super centrifuge and extracted with one-tenth volumes of cold water with mechanical stirring to effect complete dispersion. Suspensions of alcohol precipitates were usually viscous and cloudy, but standing at 2° C. for one to three days effected a settling out of an inactive floculent precipitate to give a clear solution.

Recoveries of 80 percent total activity were obtained after the usual precipitation with equal volumes of 95 percent ethanol under the low temperature conditions given.

Adsorption on starch

Macerans amylase was found to be readily adsorbed on native starch granules from solution in dilute alcohol and acetone. It could be readily eluted from the starch by washing with dilute aqueous solution of a soluble substrate.

This procedure was a modification of one reported by Holmbergh (44) for separation of the malt alpha and beta-amylases.

Potato starch was defatted by repeated extraction with boiling 80 percent methanol. This starch was further extracted with portions of 25 percent acetone until centrifugation gave a clear supernatent liquid, free from suspended starch.

Clear solutions of alcohol-precipitated enzyme in 25 percent acetone were stirred with this pretreated starch for periods of one-half to one and a half hours at 2° C. The adsorption capacity of the starch for the enzyme was not determined, but one gram of starch was found sufficient to adsorb at least 300 units of enzyme from 25 percent acetone solution.

After adsorption, the starch was centrifuged down and washed two times with 25 percent acetone. Elution of the active enzyme was effected by stirring for one-half to one hour in the cold with 0.1 to 0.5 percent Schardinger beta dextrin solution. The amount of eluant was arbitrarily set at one-tenth the original bacterial filtrate volume. Beta dextrin was found to interfere with the enzyme assay if present in the activity determination over one percent. It could be effectively removed from enzyme solutions by dialysis in the cold.

Dialysis and pervaporation

The purest macerans amylase preparations obtained were found to be stable to dialysis in the cold up to 48 hours. After that time, the activity would decrease slowly.

Concentration of the purified enzyme solution was effected by pervaporation, or evaporation in a cold air stream from a viscose tubing bag. In the presence of Schardinger beta dextrin, the enzyme was stable indefinitely; in the absence of a protective substrate, activity was rapidly lost on pervaporation.

Thus, enzyme solutions were most effectively concentrated by pervaporation in the cold of the beta dextrin eluates, followed by dialysis against hourly changes of cold distilled water for a period of eight to twelve hours.

Purification procedure

The purest macerans amylase preparations obtained were subjected to the following steps: alcohol precipitation, adsorption on starch, elution with beta-dextrin solution, dialysis, readsorption, reelution, pervaporation and dialysis. Relative degrees of purification were determined by nitrogen or tyrosine analysis on the assayed enzyme preparations. Table II shows a typical enzyme purification.

Table II. Purification of Crude *macerans* amylase.

Enzyme Solution	Percent Original Activity	Micrograms Nitrogen per Unit Activity
Bacterial filtrate	100 percent	126
50 percent alcohol precipitate	80 percent	40
β-dextrin eluate from starch	60 percent	0.25

Characterization of *Macerans* Amylase

Hydrolytic activity

The hydrolytic activity of *macerans* amylase preparations was reduced by greater purification. Further, the hydrolytic activity was shown to be preferentially inactivated by heating with the purer, substrate-free preparations.

Aliquots of an enzyme preparation, which had been alcohol precipitated, eluted from starch with beta dextrin solution, and dialyzed, were heated at 65° C. for varying periods of time. The *macerans* activity was found to decrease gradually. The preheated enzyme aliquots were then digested with equal quantities of amylodextrin substrate

for periods corresponding to 500 conversion periods each. The reducing power of the digests was found to be less in those digests of enzymes which had been heated longer. The results are given in Table III.

Table III. Change of Reducing Values of Comparable Amylodextrin *macerans* Digests after Preheating of Enzyme.

Time of Heating at 65° C.	Enzyme Activity	100 C.P.	R_{Cu} of Digest
0 minutes	15 u/ml.	150 minutes	375
5	12	180	322
10	7	280	308
15	3	660	308*

*Digested for 113 instead of 100 conversion periods.

Here, the final R_{Cu} value may be the limiting value or it may be high because of the longer period of digestion. At any rate, this enzyme must have a hydrolytic activity of at least 2.39 percent, expressed as percent of available glucose.

An additional starch adsorption and elution with 0.2 percent beta-dextrin solution yielded an enzyme preparation which showed an apparent lower hydrolytic activity on digestion with a soluble starch substrate for

periods up to 100 conversion periods. The results are shown in Table IV.

Table IV. Change of Reducing Value of a Starch Substrate with Digestion by Purified macerans Amylase.

Time of Digest	Change in R_{Cu} on Digestion	Percent Glucose equivalent
0 C.P.	0	0
5	21.7	.78
10	25.3	.90
50	28.9	1.03
100	32.5	1.16

If the gradual linear rise in reducing power from 5 to 100 conversion periods is extrapolated to 500 conversion periods, the hydrolytic activity would be 1.4 percent at that point, which may be compared to the value of 2.4 percent after a single starch adsorption.

Inactivation by heating

The rate of heat inactivation of macerans amylase has been found to vary greatly with degree of purity of the enzyme. One macerans amylase preparation (twice acetone precipitated) showed loss in activity after preheating at different temperatures for varying periods of time as shown in Figure 2.

In the presence of a "protective substrate", such as

beta Schardinger dextrin, the rate of inactivation by heating greatly decreases. This can be shown by the results in Figure 3, where enzyme solutions of comparable purity (alcohol precipitated and eluted from starch) were heated in the presence of varying concentrations of beta dextrin and then assayed.

Some indication of the relative rates of heat inactivation and temperature acceleration of macerans action can be concluded from Table V. Here, enzyme solutions were assayed under the standard conditions of the Tilden and Hudson assay procedure at different temperatures. All assay conditions except temperature were comparable, and the relative times necessary to reach the Tilden and Hudson endpoint are converted to "apparent activities".

Table V. Relative Rates of macerans Action at Different Temperatures.

Temperature of Assay	"Apparent Activity"
40° C.	100 percent
55° C.	180
65° C.	100
75° C.	70

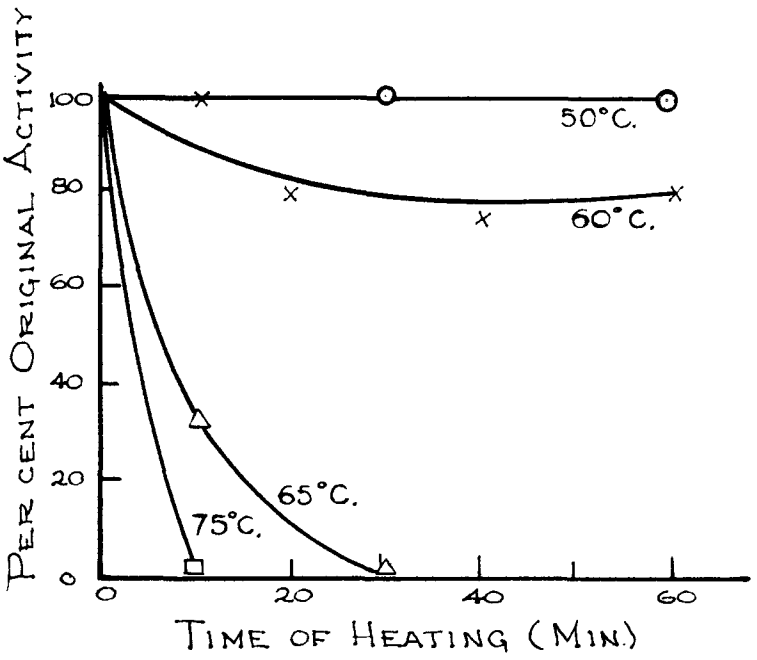

Fig. 3. Inactivation of Macerans Amylase by Heating at Different Temperatures.

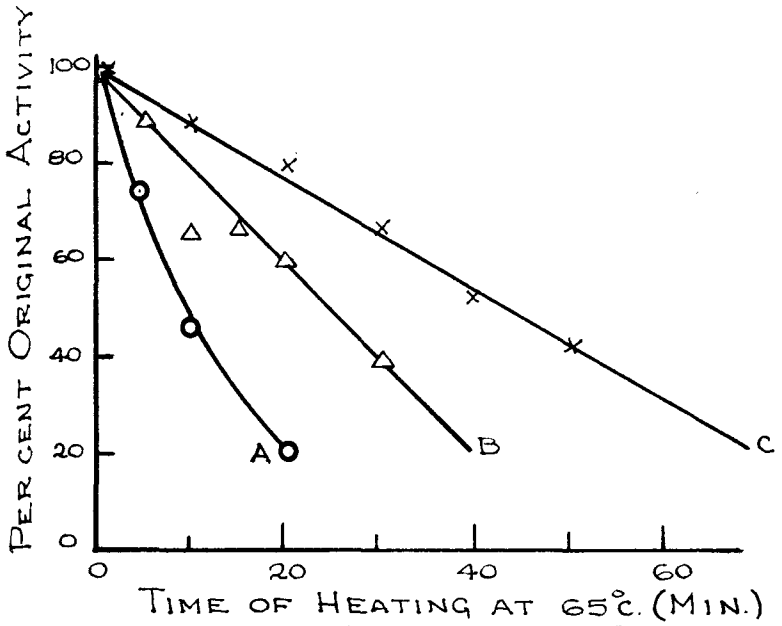

Fig. 3. Heat Inactivation of macerans Amylase at 65°C.:

A, enzyme solution alone; B, enzyme solution plus 0.04 percent β-dextrin; C, enzyme solution plus 0.2 percent β-dextrin.

Acetone precipitation at different pH values

The efficiency of acetone precipitation at different pH values was determined to provide information on the stability characteristics of the enzyme protein. A solution of an acetone precipitate (the 33 to 50 percent acetone fraction) at 20 units per ml. was adjusted to varying pH values with 0.03 N HCL and 0.03 N NaOH. Aliquots were withdrawn at desired pH intervals and 0.05 M NaCl and 30 percent acetone added. The precipitate was dissolved in an equal volume of water and assayed; the filtrate was freed from acetone by evaporation under vacuum in the cold and assayed. The activity distribution between filtrates and precipitates is shown in Figure 4.

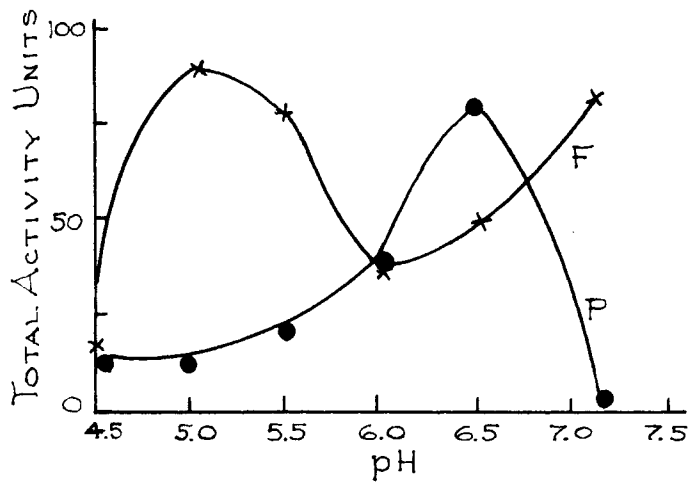

Fig. 4. Effect of pH on Precipitation of macerans Amylase with 30 percent Acetone:

F, total filtrate activity; P, total precipitate activity.

Inhibition studies

Reagents specific for sulfhydryl groups, carbonyl groups, and divalent cations were tested as possible inhibitors of *macerans* amylase activity. In Table VI are shown the results of enzyme treatment with neutralized reagents.

Table VI. Inactivation of *macerans* Amylase by Specific Inhibitors.

Inhibitor	Percent Original Activity	
	.004 M Inhibitor (16 hours at 2° C.)	.01 M Inhibitor (5 hours at 25° C.)
None	100	100
Phenylmercuric chloride	100	30
Iodoacetic acid	100	100
Phenylhydrazine	0	0
Semicarbazide	100	100
Hydroxylamine	100	0
Sodium oxalate	100	100
Sodium fluoride	80	100
Ethylenediamine tetraacetic acid	-	100

Heavy metal cations were also tested as enzyme inhibitors. For those metals showing inhibition at 0.005 M concentration, reversibility of this reaction was tested

by the addition of cyanide and cysteine. Results are given in Table VII.

Table VII. Inactivation of macerans Amylase by Heavy Metal Cations.

Salt (.005 M)	Residual Activity	Precipitate Formation	Reactivation .03 M NaCN	Reactivation .013 M cysteine
$Pb(Ac)_2$	100 percent	+		
$Hg(Ac)_2$	0	-	+	10 percent
$AgNO_3$	0	+	tr.	50 percent
$Cu(Ac)_2$	0	+	+	0
$Ba(NO_3)_2$	100 percent	±		
$FeCl_3$	0		-	0
$ZnCl_2$	0	0	+	90 percent

In the course of working with macerans amylase, it was also noted that the enzyme continued to act upon starch after the addition of iodine under the conditions of the Tilden and Hudson assay. This showed it to be stable to 0.025 N iodine in 0.025 M potassium iodide at room temperature. It was of interest to note, in addition, that aeration of B. macerans cultures at 37° C. for long periods of time increased, rather than reduced, enzyme activity in the filtrates. Reducing agents, such as

cysteine and sodium cyanide, also did not inactivate the enzyme.

Electrophoretic behavior of macerans amylase

Electrophoretic study of macerans amylase preparations has given inconclusive results because of the small amount of pure enzyme available. An attempted resolution of an active acetone precipitate at 20 units per ml. in phosphate buffer at pH 7.6 gave the patterns in Figure 5.

Fig. 5. Electrophoretic Patterns of an Active Acetone Precipitate:

Buffer 0.02 M phosphate, 0.18 ionic strength with NaCl, pH 7.6; 30 milliamps; 115 volts; 233 minutes.

Here the small peak had descending and ascending anionic mobilities of 5.5 and 5.2, respectively. The main peak

showed a mobility of 1.4 in both descending and ascending patterns.

Assay of the contents of the cell limbs at the conclusion of optical analysis allowed calculation of the mobility of the active enzyme component, according to the method of Weibull and Tiselius (45). The active component was found to have migrated with descending and ascending mobilities of 1.4, in agreement with the mobilities observed for the main slow moving peak.

Electrophoresis in a similar buffer at pH 5.7 showed no detectable resolution or migration of the acetone precipitated preparation. Studies at other pH values were not attempted because of enzyme inactivation outside the range of pH 5.0 to 7.5.

Mode of Action of *Macerans* Amylase

Heptasaccharide digest

The action of *macerans* amylase was followed on a ten percent heptasaccharide substrate. This heptasaccharide, more accurately called amyloheptaose, had been prepared according to French and co-workers (46) by controlled hydrolysis of the Schardinger beta dextrin, or cycloamyloheptaose. The digestion was carried out under sterile conditions, and aliquots removed for

analysis at intervals up to 500 conversion periods.

Five grams neutral hentasaccharide in 50 ml. water was autoclaved in a cotton-plugged separatory funnel. The enzyme solution (alcohol precipitated and starch adsorbed) was filtered with suction through a sterilized fritted glass bacterial filter. Ten ml. enzyme solution at 15 units per ml. was transferred to the substrate in a sterile pipette. After thorough mixing 6 ml. aliquots (0.5 g. substrate) were drained into sterile, calibrated, cotton-plugged tubes. All transfers were carried out aseptically in a bacteriological transfer room. The aliquot portions were incubated at 33° C. for the specified number of one-hour conversion periods, then individually removed and boiled to inactivate the enzyme.

Each aliquot was made up to 10 ml. volume and the Schardinger dextrins precipitated with trichloroethylene by shaking for 12 hours at 2° C. The Schardinger dextrin complex precipitate was then filtered off through a tared alundum crucible, dried at 70° C. under vacuum, weighed, dissolved in 10 to 20 ml. boiling water, boiled to remove trichloroethylene, evaporated to dryness in a tared beaker, and weighed again. These samples were then suitable for electrophoretic analysis after solution in 12 ml. of 0.087 M potassium iodide electrolyte. The details of the electrophoretic analysis of mixtures of Schardinger

dextrins are given in Appendix 3.

The filtrates from the Schardinger dextrin separations, containing the straight chain reducing components, were prepared for analysis by oxidation to the corresponding acids as in Appendix 1. These oxidized mixtures were analyzed electrophoretically as in Appendix 2.

The changing proportions of Schardinger dextrins in the digest aliquots are shown in Table VIII. The changing distributions of these dextrins is shown by the electrophoretic patterns of Figure 6 and the mobilities in Table IX.

Table VIII. Formation of Schardinger Dextrins from 10 percent Heptasaccharide Substrate.

Conversion Periods	Trichloroethylene Precipitate	I_3^- Test for Schardinger Dextrins	Calcium Salts Oligosaccharide Fraction
¼	.0350 gm.		
½	.0397		.3645 gm.
1	.0358	α (tr)	(.1484)
3	.0630	α	.3249
9	.0873	α, β	.3026
61	.1170	α (tr), β	
100	.1067	α (tr), β	.2454
270	.0551	β	.2581
500	.0304	β (tr)	.3127

Fig. 6. Electrophoretic Patterns of Schardinger Dextrin Fractions of a 10 percent Heptasaccharide Digest:

Analyses at 3, 9, 100, and 500 conversion periods; Descending and ascending patterns for each.

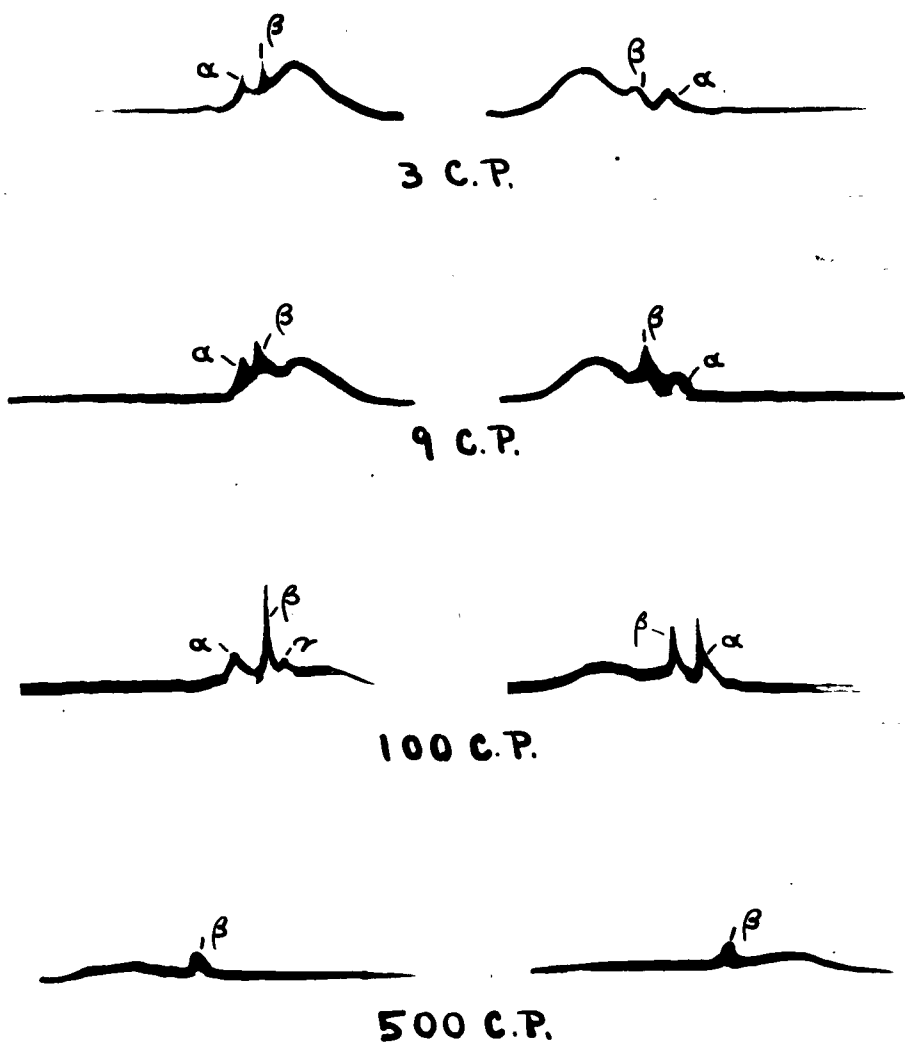

FIG. 6

Table IX. Mobilities of Peaks from Schardinger Dextrin Fractions of macerans-Heptasaccharide Digest.

Time of Digest	Mobilities (cm/sec/V/cm x 10^5)					
	α-dextrin		β-dextrin		γ-dextrin	
	Desc.	Asc.	Desc.	Asc.	Desc.	Asc.
3 C.P.	2.7	3.3	2.2	2.6		
9 C.P.	2.7	3.1	2.3	2.4		
60 C.P.		3.0	2.0	2.3		
100 C.P.	3.1	3.3	2.4	2.7	2.1	2.2
270 C.P.			1.9	2.4	1.6	2.2
500 C.P.			2.0	2.3		

The changing composition of the oligosaccharide fractions of the enzymolysis aliquots is shown in Table X and the ascending electrophoresis patterns in Figure 7.

It should be noted here that the long period of time of incubation for those aliquots reacting beyond 100 conversion periods allowed appreciable evaporation from the cotton-stoppered tubes. At 500 conversion periods (three weeks) the aliquots had decreased in volume approximately 40 percent. At the high substrate concentration this was accompanied by a deposition of a semi-crystalline polysaccharide. This polysaccharide stained dark red with iodine, with an adsorption maximum at 750 λ. The original

heptasaccharide adsorption spectrum maximum was at about 650 λ.

Table X. Mobilities of Peaks from Oligosaccharide Fractions of macerans-Heptasaccharide Digest.

Time of Digest	Ascending Mobilities (G_1	G_2	G_3	G_4	G_5	G_7	G_{7+n} (Cm/sec./V/cm. x 10^5)
¼ C.P.		9.9		7.3		4.8	4.0
½		9.8		7.0		4.8	3.9
1				7.4		4.3	
3				7.3	6.7	5.2	3.9
9		10.3		6.8	6.0	5.2	3.8
100		10.4	9.1	7.7			
270		10.5	8.2	7.0			
500	11.1	10.2	8.2	7.1	6.4		

The deposited particles appeared as spherocrystals under the microscope and exhibited a dark cross between crossed nicols. In general, this polysaccharide closely resembled amylodextrin preparations from starch.

Fig. 7. Electrophoretic Patterns of the Oligosaccharide Fractions of a 10 percent Heptasaccharide Digest:

Analyses at 1/4, 1/2, 1, 3, 9, 100, 270 and 500 conversion periods; ascending patterns only.

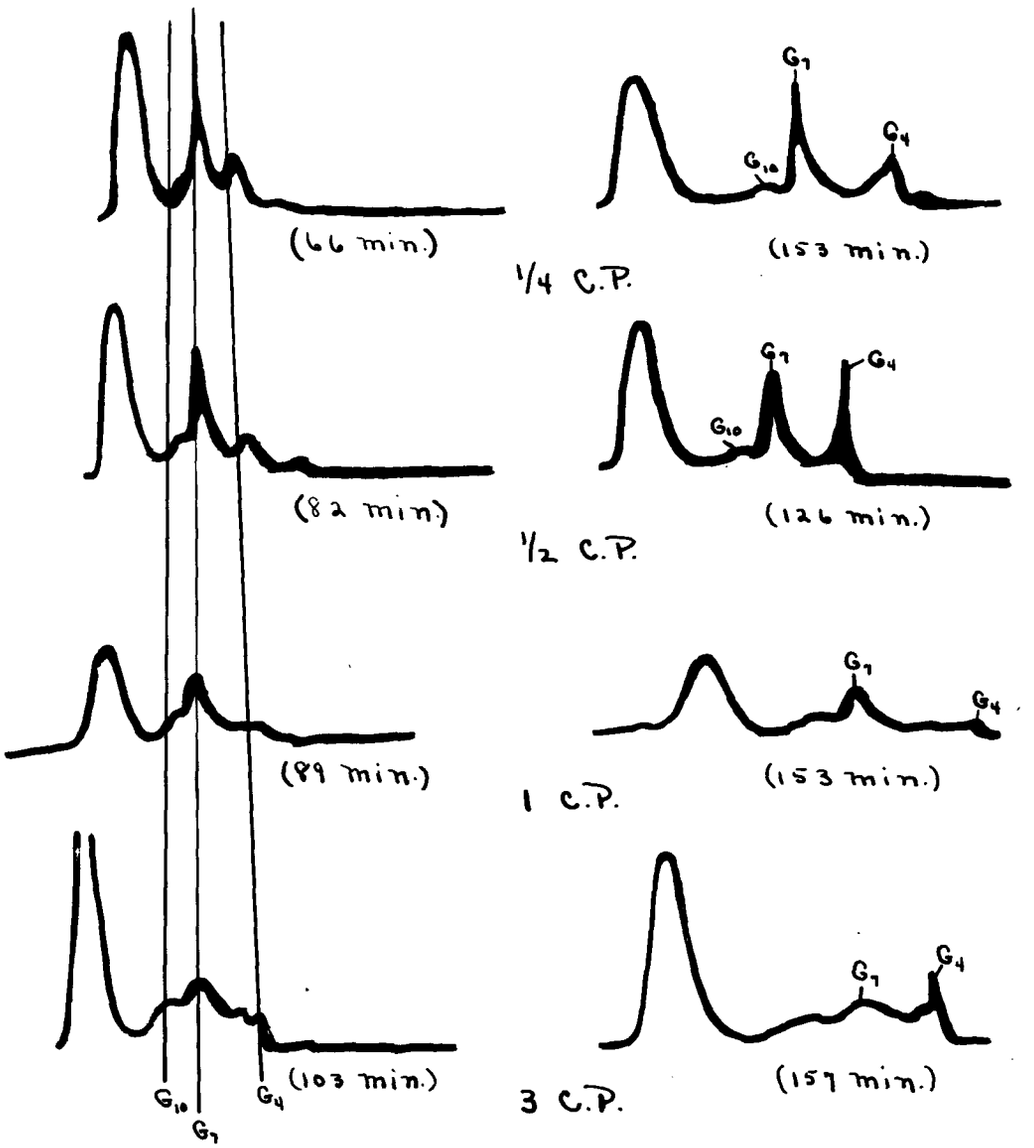
(66 min.)
1/4 C.P.
(153 min.)
(82 min.)
1/2 C.P.
(126 min.)
(89 min.)
1 C.P.
(153 min.)
(103 min.)
3 C.P.
(157 min.)
G10
G7
G4

FIG. 7

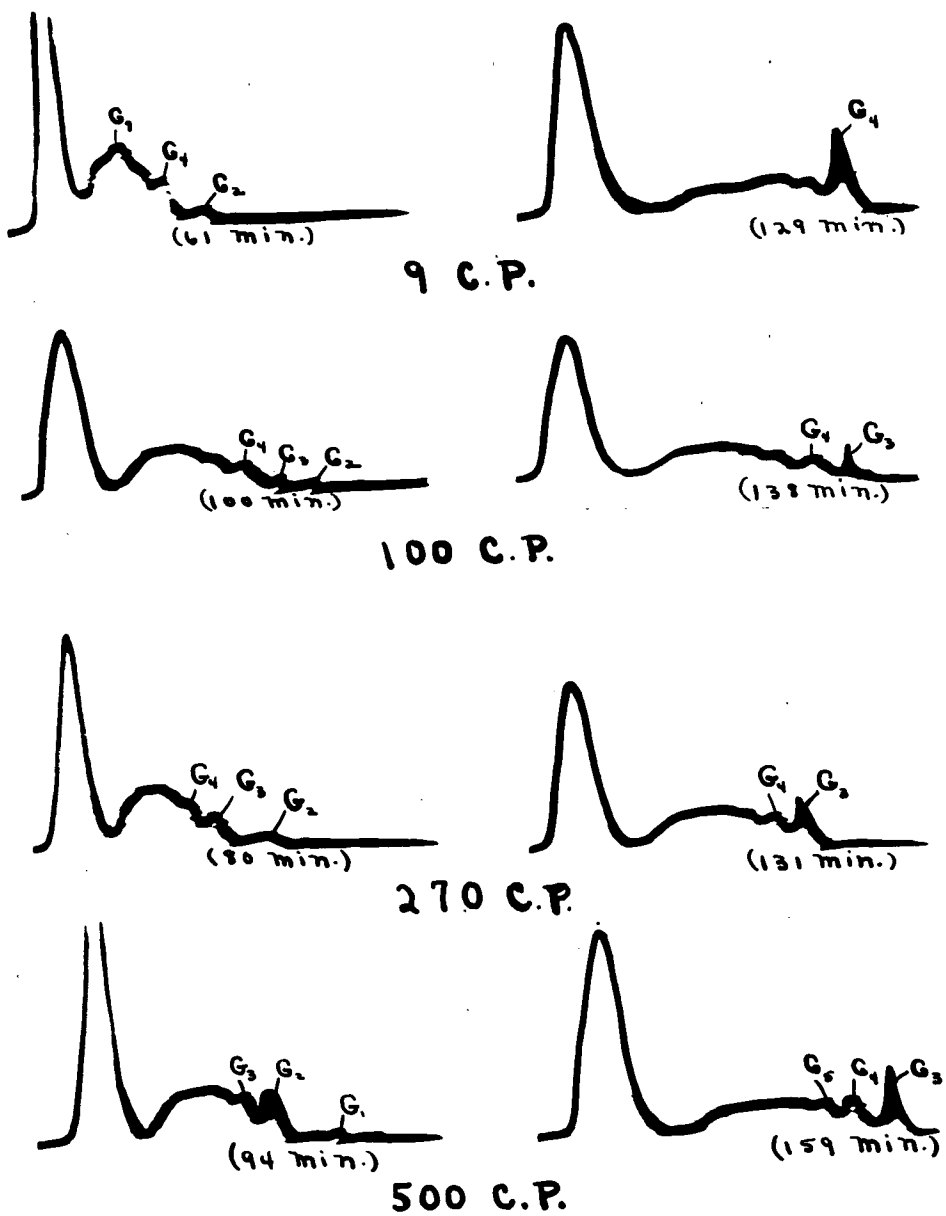
G1
G4
G2
(61 min.)
G4
(129 min.)
9 C.P.
G4
G3
G2
(100 min.)
G4
G3
(138 min.)
100 C.P.
G4
G3
G2
(80 min.)
G4
G3
(131 min.)
270 C.P.
G3
G2
G1
(94 min.)
G6
G4
G3
(159 min.)
500 C.P.

FIG. 7 (cont.)

Alpha dextrin and glucose substrate digest

The course of action of macerans amylase on a substrate of Schardinger alpha dextrin and glucose was followed. The change in reducing value of a one percent digest (0.0025 M in glucose and alpha dextrin) was followed by determining R_{Cu} values on digest aliquots by the alkaline ferricyanide method of Farley and Hixon (47). The increase of R_{Cu} with digestion time is shown in Figure 8.

Analyses of glucose concentrations during the course of the digest were run concurrently with reducing value determinations. Glucose was determined by a colorimetric method following a modified Barfoed procedure (48). The glucose concentration was found to decrease as the R_{Cu} values increased. These results are shown in Figure 9.

Equilibrium studies

The course of macerans amylase action was followed in parallel digests of different substrates of comparable concentrations. The changes in composition of three substrates were followed: heptasaccharide, alpha dextrin plus glucose, and beta dextrin plus maltose. Each substrate was 0.0025 molar in reducing saccharides and contained a one percent total carbohyrate concentration.

Digests were conducted under sterile conditions.

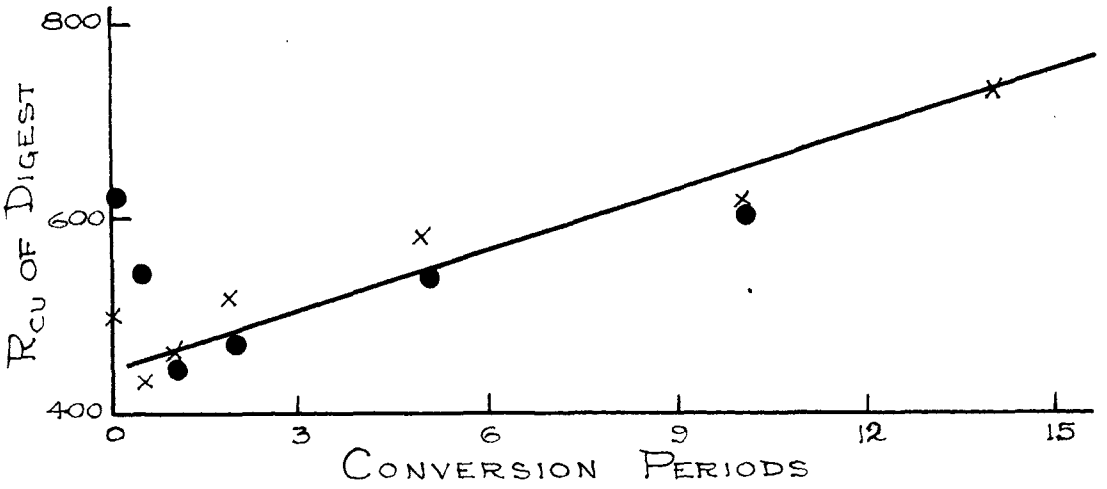

Fig. 8. Change of Reducing Value on macerans Amylase Digestion of Alpha Dextrin and Glucose Substrate.

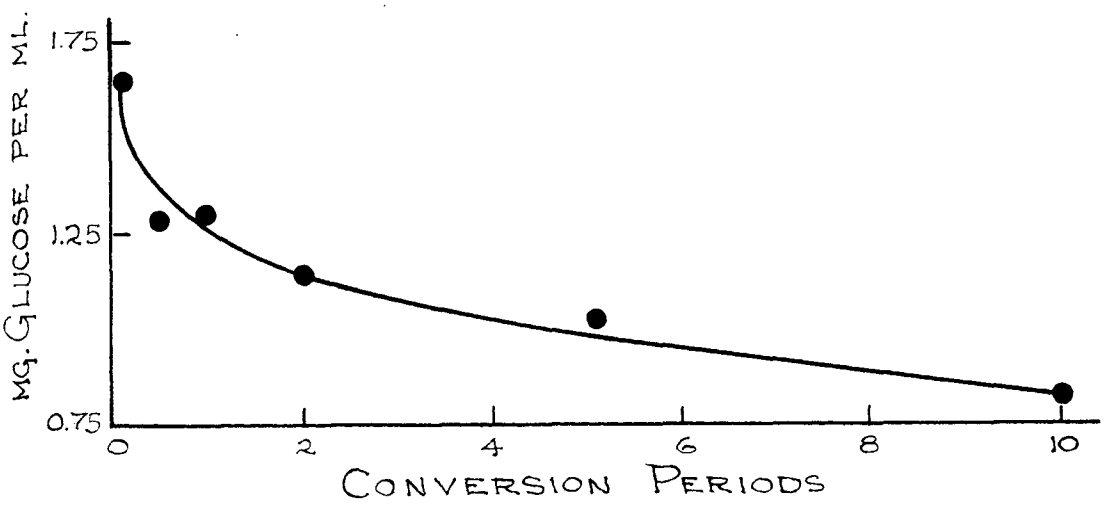

Fig. 9. Glucose Disappearance on macerans Amylase Digestion of an Alpha Dextrin and Glucose Substrate.

Substrates were autoclaved in cotton plugged flasks; the enzyme was filtered through a bacterial filter and added to the substrate in a sterile pipette. Sufficient enzyme was added to each digest to allow a conversion period of one hour at room temperature.

At specified intervals, 45 to 50 ml. aliquots were removed from the digests and made up to 50 ml. volume. Five ml. aliquots were removed from these for reducing value determinations, and the remaining 40 ml. portions evaporated to dryness in tared beakers to determine exact carbohydrate concentration. The dried residues were then dissolved in 15 ml. water and the Schardinger dextrins precipitated with trichloroethylene, by shaking at room temperature for 12 hours and standing at 2° C. for 12 hours. The weights of Schardinger dextrin-trichloroethylene precipitates were determined by filtering through tared alundum crucibles and drying at 80° C. under vacuum. The filtrates, containing the reducing components, were prepared for electrophoretic analysis by hypoiodite oxidation, as described in Appendix 1.

The changes in reducing value and Schardinger dextrin content of the three concurrent digests are shown in Figures 10 and 11.

Analyses of the oligosaccharide fractions of the

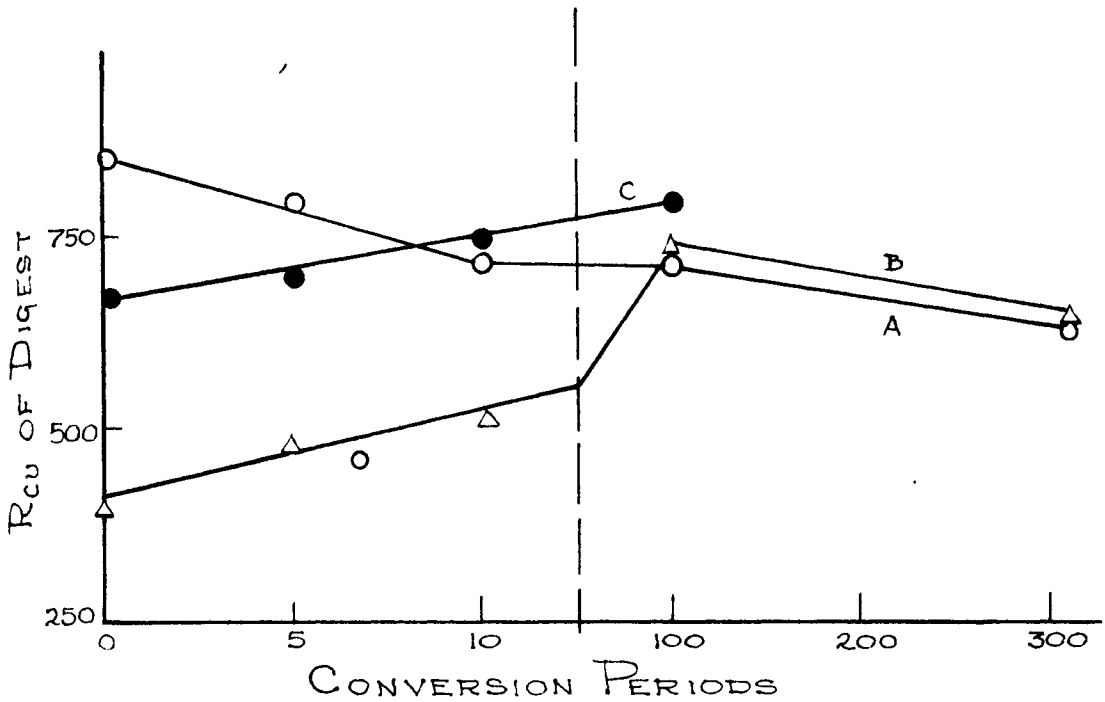

Fig. 10. Change of Reducing Value of macerans Amylase Digestion:

A, heptasaccharide; B, alpha-dextrin and glucose; C, beta-dextrin and maltose.

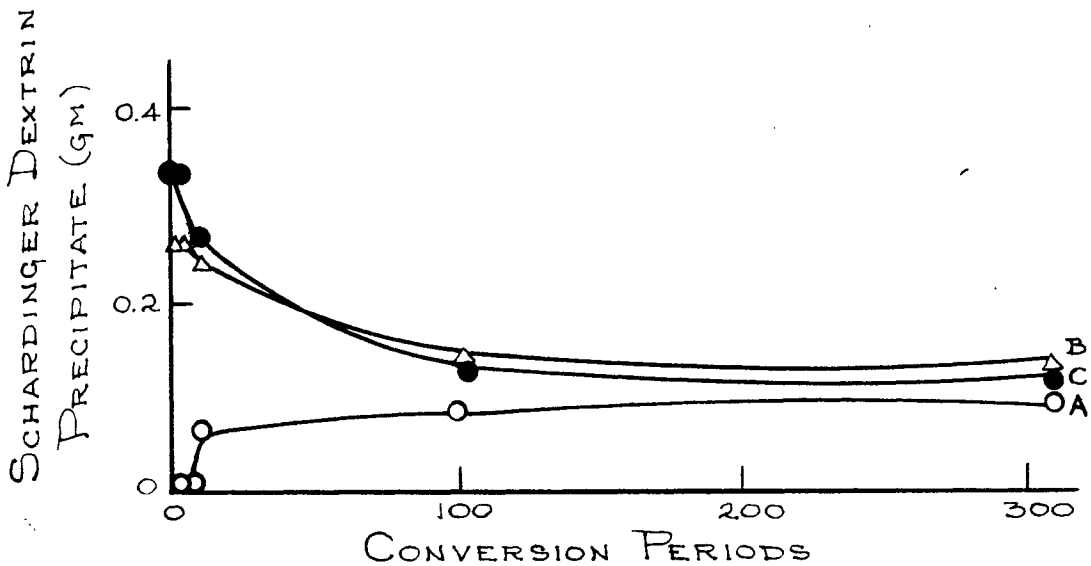

Fig. 11. Schardinger Dextrin Recoveries in macerans Amylase Digests:

A, 0.5 gm. heptasaccharide substrate; B, 0.5 gm. alpha-dextrin and glucose substrate; C, 0.5 gm. beta-dextrin and maltose substrate.

d[illegible]ts at 100 and 300 conversion periods are shown in t[illegible]lectrophoretic patterns of Figure 12. No analysis w[illegible]nade of the beta dextrin and maltose enzymolysis t[illegible]nd 100 conversion periods, due to contamination of t[illegible] digest.

Maltose digest

The action of macerans amylase on a substrate of recrystallized maltose was followed by change in rotation. A 5 percent substrate of dialyzed, recrystallized maltose was incubated at 40° C. with sufficient enzyme for a six minute conversion period in an all-glass polarimeter tube under toluene. The digest remained clear during the course of the reaction. The decrease in specific rotation during the course of the enzymolysis is shown in Figure 13.

No Schardinger dextrins could be detected after 1000 conversion periods, but the digest was analyzed electrophoretically for reducing oligosaccharides at that point. Figure 14 and Table XI show the results of this analysis.

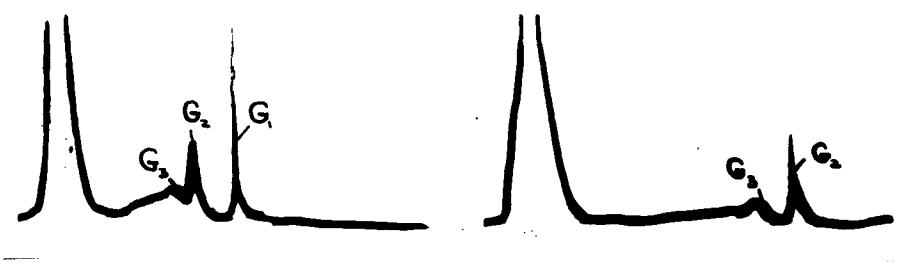

Fig. 14. Electrophoretic Pattern of a 5 percent Maltose Digest after 1000 Conversion Periods. Ascending patterns at 118 and 156 minutes.

Fig. 12. Electrophoretic Patterns of Oligosaccharide Fractions of Parallel Digests:

A, Heptasaccharide; B, alpha dextrin plus glucose; C, beta dextrin plus maltose; at 0, 100 and 300 conversion periods; ascending patterns only.

FIG. 12

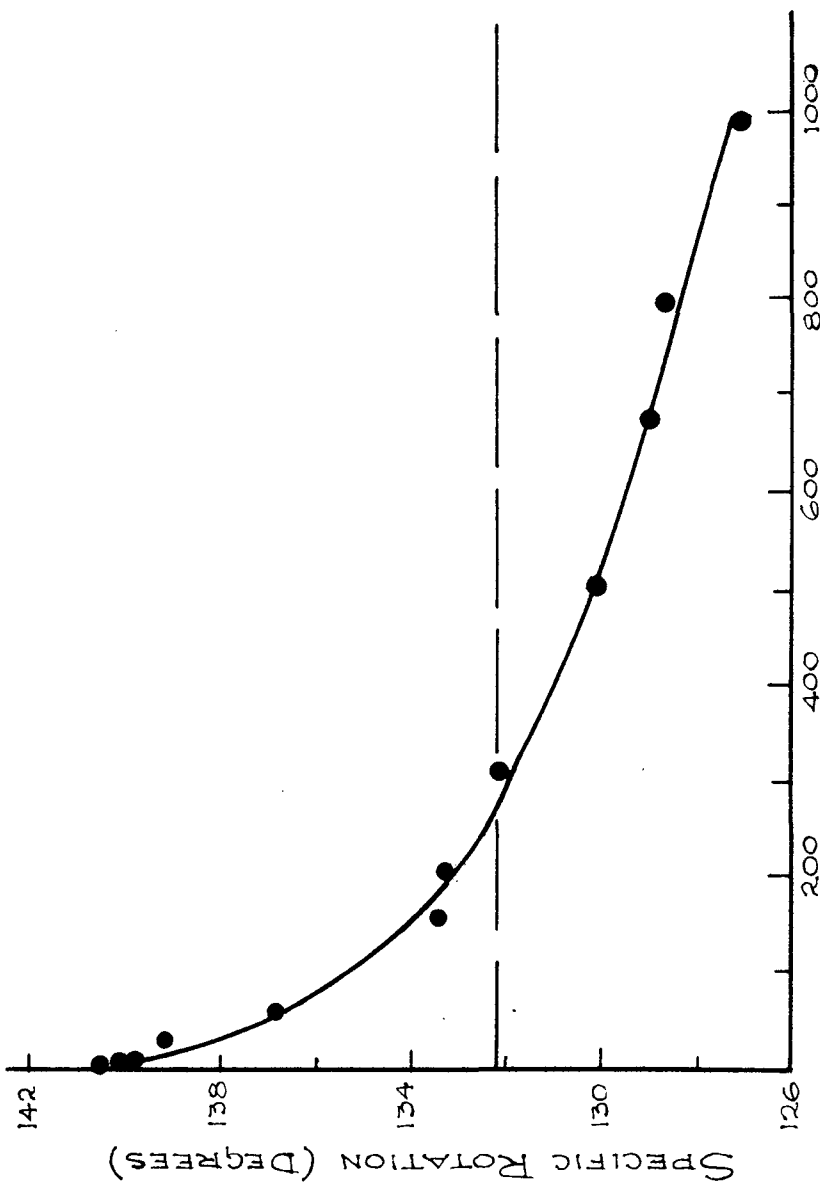

Fig. 13. Macerans Amylase Action on 5 percent Maltose Substrate.

Table XI. Electrophoretic Mobilities of macerans Amylase Digest of Maltose.

Peak	M x 10^5(cm./sec./V/cm.)	Component
I	12.0	glucose
II	9.7	maltose
III	8.4	trisaccharide
IV	7.2	tetrasaccharide

Beta-amylase limit dextrin digest

One aspect of the action of macerans amylase on beta-amylase limit dextrin was studied. It was shown that, although Schardinger dextrins were not formed from this substrate, there was an initial rapid decrease in viscosity of limit dextrin solutions on treatment with macerans amylase.

This liquefying effect was observed with dialyzed, salt-free enzyme preparations. It was further compared with the effect produced by the hydrolytic salivary amylase. Figure 15 shows the results obtained on treatment of a two percent solution of beta-amylase limit dextrin (from waxy maize) with comparable amounts of macerans and salivary amylases. Viscosity measurements were carried out at 25.2° C. in an Ostwald-Cannon-Fenske viscometer, Exax number 100. The substrate solution was introduced into the large bulb of the viscometer; the enzyme was added at zero time. Mixing was effected by shaking, and readings were taken on the solution as enzymolysis proceeded. The amounts of the two enzymes used had been adjusted to give nearly equal conversion periods for the two digests.

During the course of macerans amylase digestion of beta-amylase limit dextrin, the iodine coloration changed

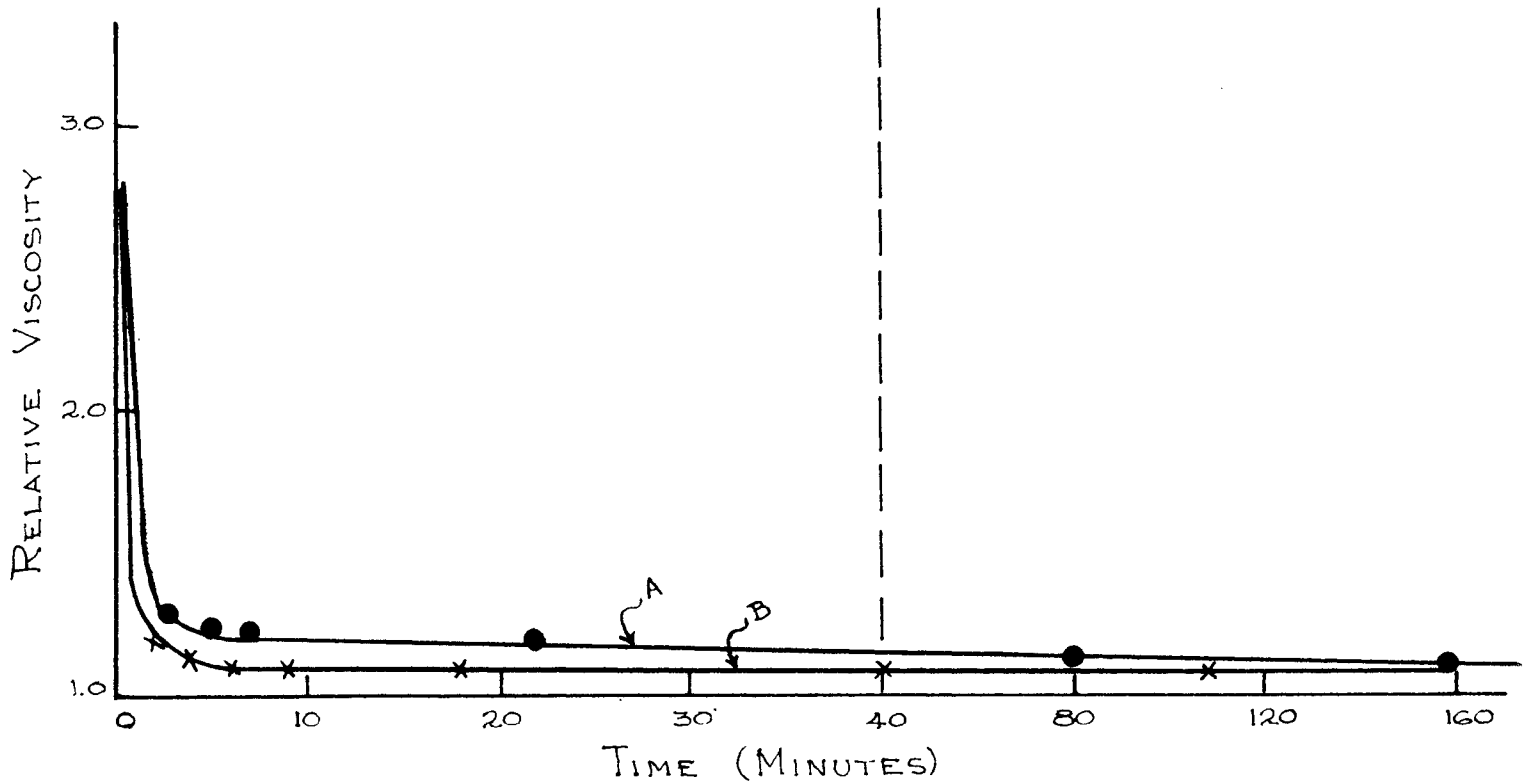

Fig. 15. Viscosity Decrease of Beta-Amylase Limit Dextrin on Enzymolysis:

A, Macerans amylase digest; B, salivary amylase digest. Both enzymes of comparable dextrinizing activity.

from lavender to brown. No Schardinger dextrins could be detected after 100 conversion periods.

Samples of limit dextrin solution were predigested with salivary amylase to a red-brown iodine coloration. The alpha-amylase was then inactivated by heating, and the substrate digested with macerans amylase for 1000 conversion periods. The iodine coloration changed from red-brown to yellow, but no Schardinger dextrins could be detected. A control digest showed that no residual alpha-amylase activity could have accounted for the loss of iodine coloration.

DISCUSSION OF RESULTS

Homogeneity of Macerans Amylase

From the results obtained in this study of macerans amylase, it seems evident that most manifestations of macerans actions may be accounted for by the catalytic function of one enzyme. The only exception to this is the slight hydrolytic action shown by macerans amylase preparations. While this hydrolytic action has not been entirely eliminated, it has been reduced to an almost negligible degree in the purest preparations reported.

It seems reasonable to assume, then, that any hydrolytic action shown by macerans amylase is due to an alpha-type amylase impurity. This would not be unexpected, since organisms similar to B. macerans, i.e. B. polymyxa and B. subtilis, are known to elaborate alpha-type amylases of high hydrolytic activity (40). The selective inactivation of hydrolytic activity over typical macerans activity by heating purified preparations at 65° C., is further indication of the presence of a dissimilar protein enzyme.

The fact that this latter effect was not observed in these studies with less purified preparations is a possible explanation for its past identification with macerans activity. Myrbäck and Gjorling (23) have reported time

studies on the hydrolytic activity of a crude B. macerans filtrate. They have shown that the rate of increase of reducing groups in their enzymolysates follows the same course as the action of malt alpha-amylase. It is possible that their bacterial filtrate may have contained a hydrolytic amylase elaborated by B. macerans, together with a relatively low concentration of macerans amylase: they reported 2.4 percent hydrolysis after one 48-hour conversion period to a brown iodine color.

McClenahan, Tilden and Hudson (35) have reported their purest preparation to promote 1.64 percent hydrolysis, determined by reducing power, after a digest time equivalent to 100 conversion periods. This preparation had been purified by acetone precipitation and adsorption on aluminum hydroxide. It can be compared with a preparation subjected to low temperature alcohol precipitation and two adsorptions on starch. The relative rates of hydrolysis of a starch substrate by the two enzyme solutions are shown in Figure 16.

Here, the initial 0.75 percent rise in reducing power during the first five conversion periods with our preparation is not readily explainable. It may be due to inaccuracies of the method of reducing value determination. This question requires further study, but the immediate importance of Figure 16 lies in the relative slopes of the two plots of increase of substrate hydrolysis with time.

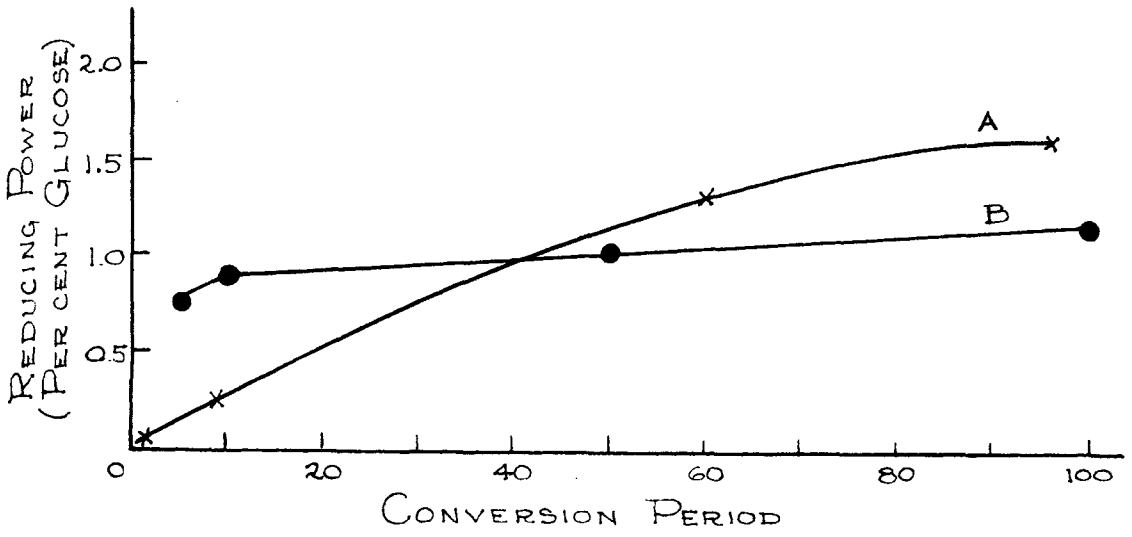

Fig.16. Rates of Increase of Reducing Power of macerans Amylase Starch Digests:

A, Tilden and Hudson's purified enzyme;
B, Enzyme purified by two adsorptions on starch.

Nature of the Macerans Enzyme

Charge on molecule

The nature of the macerans amylase protein has been clarified to a small degree. The lack of sufficient enzyme for working with high concentrations, i.e. one to two percent solutions, of purified enzyme protein has made conclusive results from electrophoretic study impossible.

The failure of the active component in our crude preparation to migrate independently may be due to low charge on the enzyme molecule or to interaction with the large amount of contaminating protein present in the slow-moving peak.

The results obtained by acetone precipitation of an active fraction at different pH values may be impossible to interpret for similar reasons. From the data given in Table I, it can be calculated that the protein concentration of the enzyme solution used was about 0.5 percent, but the concentration of the enzyme protein component could have been no greater than 0.003 percent. This shows that the relative activity of the enzyme fraction was so low that independent characteristic action of this component might be hindered. All that can be said with assurance is that the enzyme component separated out with an excess, inactive protein component whose solubility reached a minimum at pH 6.0 to 6.5. If this is a reflection of the nature of the enzyme molecule, it would seem to indicate that the net charge on the protein molecule would be at a minimum in this pH range. This effect would be magnified by the addition of acetone to the aqueous solvent to lower its dielectric constant.

Much is to be hoped for from further studies on higher concentrations of the purified enzyme protein. In the presence of large excesses of inactive protein, the

physical properties of the macerans amylase component cannot be specifically determined.

Active centers

Inhibition studies have also given inconclusive results in attempts to clarify the chemical nature of "active centers" of the macerans amylase molecule. However, it can be said that tyrosine hydroxyl groups do not appear to be essential to macerans amylase activity because of its stability to iodine. Calcium ions and other divalent ions also do not seem to be associated with the macerans enzyme, because of its stability to calcium precipitants, the cation complexing agent ethylenediamine tetraacetic acid, and dialysis.

Sulfhydryl reagents have not given completely consistent results under the conditions used. While phenylmercuric choride has shown partial inhibition, iodoacetic acid has not. While heavy metal ions inactivate, mild oxidation is relatively ineffective. The best evidence for inactivation by a sulfhydrylreaction would seem to be the reversible reaction with heavy metal ions. However, the possibility of these cations reacting with amino, amide or carboxyl groups cannot be completely disregarded.

The nature of the essential groups of some other amylases has been more completely studied. Both beta-

amylase (49) and the alpha-amylase of B. subtilis (50) have been shown to be sulfhydryl dependent. Beta-amylase is also tyrosyl dependent (51). The studies on the bacterial alpha-amylase showed a reversible inactivation with heavy metal ions very similar to that shown by macerans amylase. However, pancreatic alpha-amylase gives no evidence of sulfhydryl dependence (52).

Like macerans amylase, the subtilis amylase was further shown to be inhibited by carbonyl reagents (50). This effect has also been observed with other enzymes, as well as with these amylases, but there has been no explanation offered on the basis of known protein reactions. The possibility of a type of competitive inhibition by carbonyl reagents with amylases cannot be disregarded, since carbonyl groups are involved in the substrate linkages attacked. But the observation of similar effects with proteolytic enzymes (53) might point to the need for a more general explanation for this type of enzyme inhibition.

No studies were carried out here with reactions blocking free amino groups, although data of this nature should prove interesting. It is possible that further inhibition studies with macerans amylase might disclose some more striking correlation with the data available for other amylases. If this were true, some insight might be gained

into the inter-relation between amylase "active centers" and substrate points of attack, α-1,4-glucosidic bonds.

Denaturation

The heat inactivation studies reported here have served mainly to emphasize the protective influence of substrate on an enzyme protein. Tilden and Hudson (18) have reported complete *macerans* amylase stability to heating at 70° C. for 15 minutes based on relative rates of reaction on starch at that temperature. This has not been found to be true for heating the enzyme in the absence of substrate. The "apparent activities" shown in Table V serve to demonstrate that *macerans* amylase stability to heating cannot be accurately measured in the presence of substrate. Here, the increased rate of reaction at the higher temperature can counteract the inactivating temperature effect.

Macerans Action on Straight Chain Substrates

Heptasaccharide digest

The results of the electrophoretic studies on the heptasaccharide digests show that "redistribution" reactions, among straight-chain components proceed at a much faster rate than cyclic dextrin formation. Figure 7 shows a predominating concentration of tetrasaccharide,

heptasaccharide, and a higher saccharide during the course of the first conversion period. This would seem to point to an initial preferential attack by the enzyme on the excess heptasaccharide molecules, at the third bond in the chain. This might be expressed as in equation (1), or pictured diagramatically as in Figure 14.

(1) $2G_7 \longrightarrow G_4 + G_{10}$

(2) $G_{10} \longrightarrow G_4 +$ α -dextrin

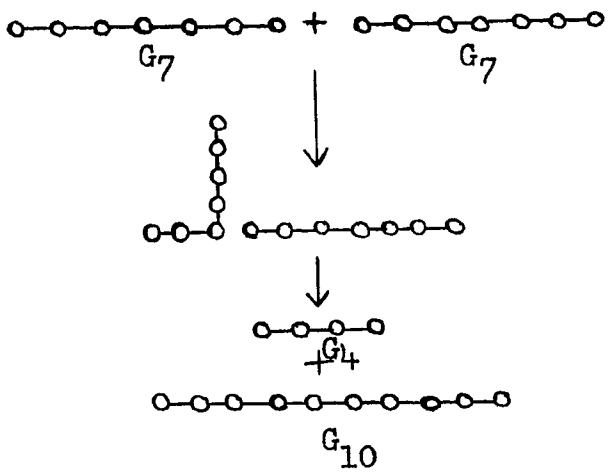

Fig. 14. A Redistribution Reaction with Heptasaccharide: Circles represent glucose residues and dashes represent α -1,4-glucosidic bonds.

Reaction (2) would account for the smaller size of the higher saccharide peak and the increasing size of the tetrasaccharide peak.

This enzyme "preference" for an innermost linkage of a

short chain dextrin is not without precedence. Myrbäck (23) has shown that malt alpha-amylase acts on starch at a rapid rate up to 15 percent hydrolysis and then levels off to a slow rate of attack. It would seem that the enzyme affinity is greater for longer chain substrate. This points to the possibility that the outermost linkages of a chain are less accessible to enzyme attack than the inner linkages. Since the nature of "redistribution" reactions requires that macerans amylase attacks chain interiors, this correlation with alpha-amylase seems justified.

It should be emphasized that the reaction of equation (1) would seem to be preferred in the early stages of the reaction in the presence of excess heptassacharide. But this reaction would not be catalyzed completely exclusive of other redistribution reactions. The nature of the reducing dextrin distribution which is approached on extended macerans action shows a random "bond exchange". That is, redistribution reactions among all dextrin species present must be occurring simultaneously.

The formation of Schardinger dextrins would seem to be only one manifestation of macerans amylase bond-exchanging activity. It appears that these cyclic dextrins are formed only after longer chains have been synthesized from the heptasaccharide substrate. This may possibly be due to the greater accessibility of two bonds in a longer chain

so that they might be joined to form a ring. This may also be a function of the postulated enzyme "preference" for inner bonds of a chain. At any rate, maximum Schardinger dextrin formation would not seem to be a final phase of macerans amylase action.

Figure 6 shows that the amount of alpha dextrin passes through a maximum before 50 conversion periods and then finally disappears altogether. Similar phenomena have also been observed by Tilden and Hudson (35) and Myrbäck (23). Table VIII also shows that the total amount of Schardinger dextrins which may be precipitated with trichloroethylene passes through a maximum and then decreases beyond 100 conversion periods.

It has been shown that hydrolytic activity for a macerans enzyme preparation purified by alcohol precipitation and one adsorption on starch should be approximately 2.5 percent at 500 conversion periods. If all of this hydrolytic action were exercised on the cyclic dextrin fraction of this heptasaccharide digest, it would not be sufficient to account for the decrease in Schardinger dextrin recoveries beyond 100 conversion periods. Therefore, the cyclic dextrins must have entered into reaction with straight chain cosubstrates to form new long reducing chains.

It has been mentioned that a polysaccharide, staining

dark red with iodine, separated out of solution in semi-crystalline form on gradual evaporation of the enzymolysates with time. This precipitation from solution of the long chain molecules must upset the attainment of equilibrium composition, and might therefore offer an explanation for the disappearance of the Schardinger dextrins. No similar enzymolysates at higher dilution were carried out; so it cannot be said whether the beta Schardinger dextrin constitutes a sizeable fraction of the equilibrium composition of a heptasaccharide enzymolysate. The possibility exists that the presence of appreciable amounts of any of the cyclic dextrins might constitute only a temporary phase in the attainment of equilibrium.

The electrophoretic analyses of the oligosaccharide fractions of the heptasaccharide digest probably provide the best indication of the nature of the equilibrium being approached. The distribution among the different glucose polymers becomes more widespread with time, until all members from glucose to iodine-staining dextrins are present. This very slow formation of maltose, and then glucose, is another example of macerans preferential affinity for the inner bonds of longer chains. These observations also offer a logical explanation for the very slow formation of fermentable sugars reported by other workers (23) (40).

Maltose digest

The course of macerans amylase action on maltose, as measured by rotational changes, follows closely that calculated on the assumption of "redistribution" reactions. Obviously, each reaction between two molecules of maltose here must give rise to one molecule of glucose. On the basis of this assumption the theoretical drop in rotation for the digest has been calculated, and is designated by the broken line in Figure 12.

The drop in rotation below the calculated value might be explained on the basis of hydrolytic contamination in the enzyme. The slope of the curve from 500 to 1000 conversion periods may be extrapolated back to an intercept at 133.0° at zero time, and this might be taken as a measure of the hydrolytic activity. From this, it can be calculated that hydrolysis, expressed as percent glucose, proceeded to the extent of 1.1 percent during the first 500 conversion periods. This would be a most reasonable figure for the enzyme preparation, and would explain the unpredicted decrease in rotation.

Electrophoretic analysis of this enzymolysate after 1000 conversion periods shows redistribution reactions to have occurred as predicted. The qualitative picture of the composition at this point also agrees well with the latter pictures of the heptasaccharide digest. It appears

that the same type of equilibrium is being approached with both substrates. The lack of formation of Schardinger dextrins would be a natural consequence of the low concentration of longer chain saccharides present at any time in the digest. If the same equilibrium is being approached as with heptasaccharide, these chains should form very slowly. Redistribution reactions which would form longer chains must also form glucose which, in excess, would exert a reversing mass action effect.

Macerans Action on Cyclic Substrates with Linear Cosubstrates

McClenahan, Tilden and Hudson (35) and Levine (42) have reported slight or negligible macerans amylase action on the purified Schardinger dextrins. Levine (42) and French, et al. (41), however, have shown that macerans action on cyclic dextrins proceeds very rapidly if an unsubstituted α-glucoside or glucose is present as cosubstrate. The place of this type of reaction, involving opening of ring molecules, in the over-all picture of macerans action was not understood.

Levine (42) has shown that there is an increase in specific rotation during the initial stages of macerans action on alpha dextrin and glucose. The results in Figure 8 show that there is an accompanying decrease in R_{Cu} values for similar digests. This evidence points to the

incorporation of glucose in linear dextrins of higher rotation and lower R_{Cu} values in a sort of coupling reaction, as in the equation,

Alpha dextrin + glucose ----> heptasaccharide.

The results of Figure 9 substantiate this, showing that glucose does disappear in the initial stages of macerans action on the cyclic dextrin.

It appears, then, that formation of cyclic molecules, or their transformation into straight chain dextrins with cosubstrates, are both results of one reaction involving simultaneous cleavage and formation of α-1,4-glucosidic bonds. Cosubstrate molecules must be present to allow the formation of a new glucosidic bond as the ring is opened. In macerans amylase digests there must be continual formation and transformation of these ring molecules in the approach to a dynamic equilibrium.

That this is a true equilibrium has been demonstrated by the studies with parallel digests. Substrates of heptasaccharide, alpha dextrin and glucose, and beta dextrin and maltose, which are equimolar in reducing groups and of equal carbohydrate concentrations, have been shown to approach similar equilibrium mixtures at 100 conversion periods. These three equilibrium mixtures are shown to have similar reducing values in Figure 10, and similar Schardinger dextrin contents in Figure 11. Electrophoretic

analysis of their respective oligosaccharide fractions, as shown in Figure 12, also shows their compositions to be similar.

The decrease in reducing values of digests beyond 100 conversion periods, shown in Figure 10, would seem to agree with the results of electrophoretic analysis. This decrease in R_{Cu} would be a result of the approach to an equilibrium composition which includes more lower molecular weight sugars, especially glucose.

From this it would seem apparent that macerans amylase action on cyclic substrates with cosubstrates is another manifestation of the same action shown on straight chain substrates. Redistribution of the available glucose residues, in linear or cyclic molecules, proceeds toward the same equilibrium composition.

Cosubstrate studies may provide a means for securing quantitative data on specific macerans reactions. Initial reaction rates between pure cyclic substrates and pure co-substrates as glucose or maltose, should be possible to determine. Quantitative data on only a few isolated redistribution reactions might be of value in defining the complex system of macerans reactions.

Macerans Action on Branched Chain Substrates

The absence of detectable quantities of Schardinger

dextrins in macerans amylase digests of beta-amylase limit dextrin has previously been accepted as evidence that macerans amylase does not react with this substrate. The viscosity drop and the iodine color change observed here under such conditions appear to indicate that some reaction does occur.

The identification of alpha dextrin in the limit dextrin digests has been doubtful. There have been some dark blue crystals formed with iodine which may be identical with the alpha-iodine complex, but they are not entirely characteristic.

However, the drop in viscosity as shown in Figure 15 would seem to be ample proof of the degradation of the beta-amylase limit dextrin to smaller fragments. This degradation cannot be due to simple hydrolytic activity, as demonstrated by parallel studies with salivary amylase, Figure 15. The concentrations of macerans and salivary amylase preparations used were calculated to allow approximately equal conversion periods to similar red-brown endpoints with iodine. Under such conditions, the macerans preparation would have had to exhibit nearly 50 percent hydrolytic activity to account for the initial drop in viscosity on that basis alone. This would be impossible. In addition, the approach of the macerans digest to the limiting viscosity value after five minutes was very slow,

while the alpha-amylase digest had achieved the limiting value by that time.

Further, macerans amylase was not capable of converting beta-amylase limit dextrin to the achroic point with iodine. If alpha-amylase was allowed to predigest the limit dextrin to a red-brown iodine color, macerans amylase could then carry it to the achroic point. This would be expected in view of the redistribution reactions possible on the hydrolyzed substrate of shorter linear and branched dextrins.

The observations of macerans action on beta-amylase limit dextrin depart from previously held concepts on its substrate specificity. However, they correlate well with its recognized rapid liquefying action on starch pastes. This action has been demonstrated by McClenahan, Tilden and Hudson (35) with their purified enzyme showing low hydrolytic activity. It has also been observed by Myrbäck (23) and earlier workers. If the action is not due to hydrolytic activity, then the concept that macerans amylase attacks only the straight chains and the end chains of starch is untenable. This type of action would not appreciably reduce the viscosity of starch or beta-amylase limit dextrin.

Redistribution reactions may occur which would result in a simple rearrangement of the branched substrate

molecules. If it is assumed that half the glucose units in a randomly branched polymer are in the outer chains, then such a redistribution could reduce the average chain length of the inner chains of beta-amylase limit dextrin by one-half. This might alter the iodine staining properties of such a substrate, but should not appreciably decrease its viscosity.

Since the sharp viscosity decrease cannot be accounted for by simple molecular rearrangement, the formation of lower molecular weight dextrins must be postulated. If these are produced by a non-hydrolytic action, then non-reducing cyclic dextrins must be formed. Whether these cyclic dextrins, formed from a highly branched substrate, would be identical with the Schardinger dextrins or branched cyclic molecules, remains to be shown.

Since Schardinger dextrins are most difficult to identify in the digests of beta-amylase limit dextrin, it would seem that their formation would not be adequate explanation for the other effects observed during digestion. However, no evidence is yet available to show whether macerans amylase is capable of forming cyclic molecules of structures other than six to eight glucose residues in a ring.

General Observations

Studies performed with limited amounts of purified macerans amylase have given an insight into the scope of its "glucosidic exchange" (54) action. It would seem to be unique in its capacity to exchange the energy liberated in the cleavage of one α-1,4-glucosidic bond to the formation of a new α-1,4-glucosidic bond.

The nature of the alpha dextrin, which is formed first, may be a clue to the configuration of the substrate. In general, it might be said that cyclization reactions by macerans amylase have been observed on substrates which stain with iodine. These substrates must be capable of existing, at least partially, in a helical configuration. It is possible that such a configuration might be essential for macerans amylase catalysis of cyclic dextrin formation.

However, the general redistribution reaction of macerans amylase on linear chains has appeared as its most significant function. This manifestation of macerans action has presented new possibilities to be considered in the study of naturally occurring polysaccharide synthesis and degradation. These substrate studies have presented a picture of a dynamic equilibrium which can be used to supply low molecular weight sugars or to build up long chain

dextrins. While this enzyme seems to be peculiar to the Bacillus macerans organism, it exhibits a type of reaction which may now be considered as a possibility for other schemes of carbohydrase action.

SUMMARY

1. A simplified method for the preparation of a highly purified macerans amylase has been developed.
2. The hydrolytic activity of macerans amylase has been shown to be due to an alpha-amylase type of impurity. This can be reduced to a negligible amount by an adsorption procedure of purification.
3. Some physical properties of macerans amylase have been studied, but conclusive results await further study on higher concentrations of the purified enzyme.
4. A method for qualitative analysis of mixtures of linear glucosidic oligosaccharides, from glucose through amyloheptaose, has been developed. This method includes hypoiodite oxidation of the reducing sugars and dextrins and electrophoretic analysis of the corresponding acid anions.
5. A method of qualitative analysis of mixtures of alpha, beta and gamma cyclic dextrins has been developed, based on electrophoretic resolution of their iodide complexes in solution.
6. Macerans amylase has been shown to catalyze a redistribution action on linear glucosidic substrates. A "glucosidic exchange", effected by simultaneous cleavage and formation of α-1,4-glucosidic bonds,

modified an amylacous substrate by redistribution of the glucose residues among linear dextrins of longer and shorter chain lengths.

7. The reversible formation of cyclic dextrins has been shown to be one manifestation of macerans amylase "glucosidic exchange" action. Opening of the cyclic molecules is accomplished in the presence of a suitable cosubstrate to allow glucosidic bond formation simultaneous with ring cleavage.

8. Macerans amylase substrate specificity has been shown to include alpha-glucosidic polymers down to maltose, but the enzyme affinity has been shown to be greater for the inner linkages of longer chains.

9. Macerans amylase redistribution action on comparable substrates of heptasaccharide, alpha dextrin plus glucose and beta dextrin plus maltose effected the approach to a common equilibrium composition including Schardinger dextrins, glucose, maltose, trisaccharide, tetrasaccharide and higher linear saccharides.

10. Macerans amylase action on a maltose substrate effects redistribution to a mixture of glucose, maltose, trisaccharide, tetrasaccharide and higher saccharides.

11. Macerans amylase attacks beta-amylase limit dextrin with a non-hydrolytic action to produce lower molecular weight dextrins. It is suggested that these dextrins may be branched cyclic molecules.

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APPENDIX 1.

Preparation of Oligosaccharide Mixtures for Electrophoretic Analysis.

Oxidation of the enzymolysis mixtures of glucose and straight chain glucose polymers was effected by an adaptation of the alkaline oxidation procedure of Goebel[1]. Here, calcium salts were substituted for barium salts, and the dextrinic acids were thrown out of solution in acetone, a good solvent for the contaminating calcium iodide. The procedure developed was as follows:

The molar concentration of reducing groups was calculated in a 0.1 to 2.0 gram sample of sugars and oligosaccharides in a maximum volume of 20 ml. A four-fold molar excess of 0.3 N iodine in 0.15 M calcium iodide solution was added. An eight-fold molar excess of calcium hydroxide was added with stirring over a three minute period. After the oxidation had proceeded at room temperature for fifteen minutes, a twelve-fold molar excess of oxalic acid, dissolved in a minimum amount of water, was added and the reaction neutralized with an excess of calcium carbonate.

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[1] Goebel, J. Biol. Chem., 72, 809 (1927).

The solution was filtered and evaporated to 10 ml. volume at 60° C. under reduced pressure. It was then thrown into 300 ml. acetone, together with 5 ml. wash water. The acetone suspension was filtered on a Büchner funnel and put through the same filter paper several times until the filtrate was clear.

The filter paper, with precipitate, was triturated in 10 to 15 ml. water, again precipitated in 300 ml. acetone, and filtered.

The final product was a mixture of calcium salts which formed a glaze over the filter paper. This was extracted with 10 ml. 0.1 M potassium acetate and filtered.

The calcium salts of the sugar and dextrinic acids were converted to potassium salts by passing through a column of Amberlite I R-100 resin, previously saturated with potassium ions. The column was thoroughly washed with 0.1 M potassium acetate, and the sample dissolved in 0.1 M potassium acetate, was introduced. The initial column volume of solvent was discarded and the solution, plus 0.1 M potassium acetate eluate, was collected up to a total volume of 12.5 ml. Completeness of ion exchange was determined by testing the solution eluate periodically with a concentrated potassium oxalate solution to detect unadsorbed calcium.

This procedure provided a quantitative oxidation of

reducing sugars and oligosaccharides. The final solution contained the potassium salts of the corresponding acids in 0.1 M potassium acetate, and was suitable for electrophoretic analysis.

APPENDIX 2.

Electrophoretic Analysis of Oligosaccharide Acid Mixtures

Solutions of one to two percent concentrations of the potassium salts of oligosaccharide acids in 0.1 M potassium acetate were analyzed electrophoretically against 0.1 M potassium acetate.

The electrophoretic apparatus used included a three part cell, as described by Longsworth, et al.[1] and a Philpot-Svensson optical system modified to employ a 35 mm. camera. Bath temperature was 2° C. The amperage was kept constant at 0.032 amperes. At this value, the potential varied from 180 to 190 volts. Analyses were completed in one to three hours under these conditions.

Mobilities were calculated according to the equation:

$$M = \frac{\text{cm.} \times L}{\text{current density} \times \text{time}}$$

where L is the specific conductance of the medium into which migration is occuring, i.e. buffer or solution.

Figure 16 shows a tracing of the electrophoretic patterns obtained on analysis of an oxidized mixture of 0.1 g. glucose, 0.1 g. maltose hydrate, and 0.1 g. amyloheptaose.

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[1] Longsworth, Cannon and MacInnes, J. Am. Chem. Soc., 62, 2580 (1940).

Only the ascending patterns were used for mobility calculations. The sharpness of the descending peaks was sacrificed to obtain an artificial sharpening of the ascending pattern for better resolution. Table XII shows the calculated mobilities for the three components of the synthetic mixture.

Table XII. Electrophoretic Mobilities for a Synthetic Mixture of Oligosaccharides under Standardized Conditions.

Component	Ascending Mobilities$_5$ (cm./sec./V/cm. x 10^5)
Glucose	11.4
Maltose	8.6
Heptasaccharide	4.8

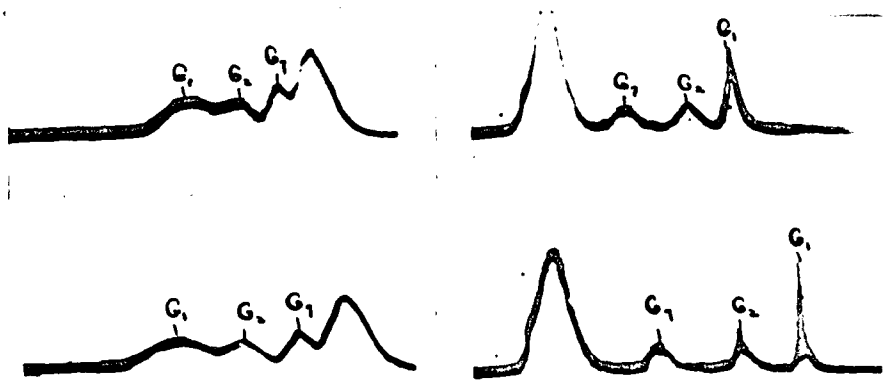

Fig. 16. Electrophoretic Patterns for a Mixture of Glucose (G_1), Maltose (G_2) and Heptasaccharide (G_7):

Descending and ascending patterns; times, 68 and 92 minutes.

APPENDIX 3.

Electrophoretic Analysis of Schardinger Dextrin Mixtures

The Schardinger dextrin mixtures, free from precipitants, have been analyzed electrophoretically in 2 to 3 percent concentration in 0.087 M potassium iodide solution against the same 0.087 M potassium iodide electrolyte. Amperage was kept at 32 milliamps and the potential, at 190 volts. Under these conditions, resolution required four to five hours.

Here, both ascending and descending patterns are sharply defined and suitable for mobility calculations. Figure 17 shows the pattern from a synthetic mixture of equal weights of alpha, beta, and gamma dextrins.

The gamma dextrin peak has never been obtained as sharply defined as the other components. This might be expected because of its weaker complexing ability with iodide.

Mobilities are calculated from the equation given in Appendix 1.

Standard mobilities, calculated from resolution of a synthetic mixture of alpha, beta, and gamma dextrins, are given in Table XIII.

Table XIII. Mobilities of Schardinger Dextrins under the Standardized Electrophoretic Procedure.

Dextrin	Mobilities (cm./sec./V/cm. x 10^5) Ascending	Descending
alpha	3.9	3.1
beta	2.8	2.4
gamma	2.3	2.2

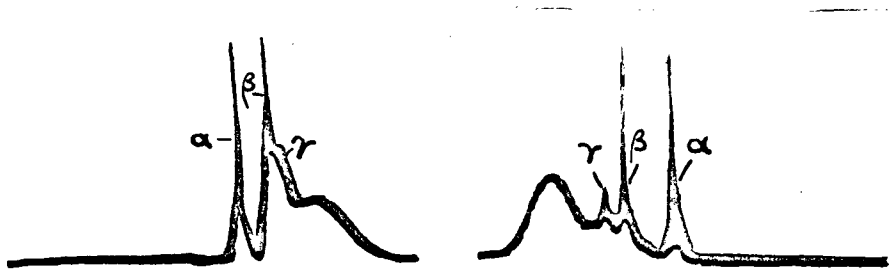

Fig. 17. Electrophoretic Patterns of a Mixture of Alpha, Beta and Gamma Schardinger Dextrins:

Descending and ascending patterns; time, 209 minutes.